DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

Contribution of somatostatin, cortistatin, their receptors and other peptides to the regulation of growth hormone in primates and mice.

José Córdoba Chacón

Córdoba 2011

TITULO: *Contribution of somatostatin, cortistatin, their receptors and other peptides to the regulation of growth hormone in primates and mice*

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Contribution of somatostatin, cortistatin, their receptors and other peptides to the regulation of growth hormone in primates and mice.

Memoria de Tesis Doctoral presentada por **José Córdoba Chacón,** Licenciado en Bioquímica, para optar al grado de **Doctor en Ciencias.**

Los Directores

Dr. Justo P. Castaño Fuentes
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Universidad de Córdoba

En Córdoba, a 20 de diciembre de 2010

DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

GRUPO DE ENDORINOLOGÍA CELULAR Y MOLECULAR

Dº Justo Pastor Castaño Fuentes y Dº Raúl Luque Huertas, Catedrático de Universidad e Investigador Contratado del Departamento de Biología Celular, Fisiología e Inmunología de la Uniersidad de Córdoba,

INFORMAN

Que Dº José Córdoba Chacón, Licenciado en Bioquímica, ha realizado bajo nuestra dirección el trabajo titulado "**Contribution of somatostatin, cortistatin, their receptors and other peptides to the regulation of growth hormone in primates and mice.**" y que bajo nuestro juicio reúne los méritos suficientes para optar al Grado de Doctor en Ciencias.

Y para que conste, firmo la presente en Córdoba, a 20 de diciembre de 2010.

Fdo.: Prof Dr. Justo Pastor Castaño Fuentes

Fdo.: Dr. Raúl Miguel Luque Huertas

TÍTULO DE LA TESIS: Contribution of somatostatin, cortistatin, their receptors and other peptides to the regulation of growth hormone in primates and mice.

DOCTORANDO/A: José Córdoba Chacón

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Durante el desarrollo de la presente Tesis Doctoral, en el periodo comprendido entre febrero de 2007 y diciembre de 2010, el doctorando José Córdoba Chacón no solo ha superado con creces los objetivos planteados al comienzo de la misma, sino que ha desarrollado y validado técnicas experimentales de una gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes en el campo de la somatostatina, la cortistatina, la ghrelina y su receptores y que quedan patentes en varias publicaciones. Concretamente, como fruto de su trabajo durante este periodo, ha publicado tres trabajos directamente relacionados con su Tesis Doctoral, en las revistas “Cellular and Molecular Life Sciences”, “American Journal of Physiology: Endocrinology and Metabolism” y “Endocrinology”, revistas de referencia dentro de nuestras áreas de investigación. Además, el trabajo realizado en este periodo ha dado lugar a otros tres artículos, que están actualmente en fase de redacción para someterse próximamente a las revistas “Plos Biology”, “Journal of Clinical Investigation” y “Endocrinology”.

Por último, el doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, de los que han derivado varios capítulos de libro.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 20 de diciembre de 2010

Firma del/de los director/es

Fdo.: Justo Pastor Castaño Fuentes

Fdo.: Raúl Miguel Luque Huertas

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, bajo la dirección de los Dres. Justo P. Castaño Fuentes y Raúl M. Luque Huertas. Dicho proyecto fue subvencionado mediante una beca del Fondo de Investigación Sanitaria del Instituto de Salud Carlos III "Ayudas predoctorales de formación en investigación en salud (FI06/00804)" concedida por el Ministerio de Ciencia e Innovación. Durante el transcurso de la presente Tesis Doctoral se han realizado dos estancias de trece y catorce semanas en el Departamento de Medicina de la Universidad de Illinois en Chicago (EE.UU) bajo la supervisión de la Dra. Rhonda D. Kineman y una estancia de trece semanas en el Departamento de Medicina Interna de la Universidad de Turín (Italia) bajo la supervisión de la Dra. Riccarda Granata. Dichas estancias fueron financiadas por el Fondo de Investigación Sanitaria del Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación.

“All men dream: but not equally. Those who dream by night in the dusty recesses of their minds wake up in the day to find it was vanity, but the dreamers of the day are dangerous men, for they may act their dreams with open eyes, to make it possible.”

T.E. Lawrence
(Seven Pillars of Wisdom: A Triumph)

“Eppur si muove”

Mal atribuida a Galileo Galilei.

Giuseppe Marc'Antonio Baretti
(Italian Library, 1757)

Agradecimientos

Sólo cuando echas la mirada al camino andado, puedes dar cuenta de la ayuda que recibiste para recorrerlo. Ahora que escribo estas palabras pienso que fueron muchas las personas, los comentarios y las impresiones que lo forjaron. Algunas de ellas, pueden que caigan en el olvido, aunque no dudo que algo de todas las experiencias vividas, al menos un resquicio, me acompañara para siempre en las decisiones venideras.

De Montemayor salí con la intención de convertirme en científico, tal vez hubiera quedado sólo en eso, en una intención, pero tuve suerte y conocí a Justo cuando empecé la licenciatura en Biología. Gracias a él, tuve la primera ocasión de entrar en un laboratorio de investigación. Sin embargo, pronto probé otras experiencias gratificantes en el departamento de Bioquímica y en el Instituto de Agricultura Sostenible del CSIC. Pero de nuevo, por "culpa" de Justo volví a Biología Celular. Cuando acabé la licenciatura en Bioquímica, recibí su apoyo que nunca faltó y del que siempre estaré agradecido para iniciar una nueva etapa como becario. Nada más empezar, me mostró la puerta de salida y me envió a Chicago donde volví a ver a Raúl al que tengo que considerar como maestro ya que me ha enseñado prácticamente todo lo que puedo hacer en el laboratorio. De él aprendí como trabajar y pensar en ciencia, como mantener un ritmo de trabajo duro y sufrir con la experimentación, pero también como disfrutar de ella y sentirme reconfortado del trabajo realizado. A ambos le estoy totalmente agradecido porque nunca podría repetir todo este trabajo, sin haber tenido su atención, rectitud e implicación en la labor que hemos realizado juntos.

En Chicago, tuve la oportunidad de conocer a Rhonda Kineman. De Rhonda guardaré siempre una impresión difícil de calificar, debido a que conseguía eclipsar todo lo que había visto e imaginado antes de una persona de ciencia. Tal vez fue una de las sensaciones más gratificantes de mi corta experiencia como investigador, ya que empezaba a recibir atención y reconocimiento de alguien que no era mi supervisor directo. Igualmente, me gustaría agradecer la atención y apoyo recibido de Riccarda Granata, que me acogió en su laboratorio.

Aunque Justo y Raul sean mis directores de Tesis y Rhonda una co-diretora de mis estancias en EE.UU. a Manuel David lo considero como un maestro más. De él empecé a aprender el día a día del laboratorio y conseguí la ayuda necesaria para obtener mis primeros resultados. Siempre ha sido una referencia y le estoy agradecido por su inestimable ayuda durante todos estos años. Al igual que Manuel, Alberto ha sido otro pilar sobre el cual pude iniciar mi labor. Siempre guardaré una gran visión de este chico que nos hizo reir con chistes malos y nos escuchó para dar otro punto de vista que la madurez y las cabezas bien amuebladas pueden ofrecer. Prácticamente durante toda la Tesis, he tenido la suerte de compartir mucho tiempo con amigos como Cristina, Alex, Sandra, Ana Q, y los incombustibles de Montemayor, Edu, Santi, Berra, Paqui, Rocio, Sergio,... a los que en estos últimos instantes de Tesis, se les sumaron Bea y Almudena y que entre todos han sido una gran "válvula de escape" en momentos difíciles.

Si muchos no lo saben, "el movimiento se demuestra andando", esta cita posiblemente datará de 400 años a.C, pero yo la pude escuchar de Antonio Jesús y le agradezco que me la transmitiera al inicio de mi Tesis porqué en momentos difíciles ha representado el estímulo necesario para seguir adelante. Aunque Antonio parece aburrirnos con historias de su vida en EE.UU. y Francia, de vez en cuando, nos ilustra con pinceladas de su dilatada experiencia.

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dejó impresionados: una capacidad de trabajo y sacrificio en el laboratorio que será difícil de superar que no puedo más que agradecer.

Muchas veces he oído decir a la gente que mis padres han tenido mucha suerte porque han tenido dos hijos que son muy listos. Tal vez la suerte no se tiene, sino que se consigue con esfuerzo. A ellos les agradezco que me enseñaran a disfrutar con lo que he hecho durante todo este tiempo, su atención y devoción. Pero sin duda, su firmeza y carácter en mi educación, su cariño y esa mano que siempre me han tendido cuando la he necesitado. Pienso que uno se parece por genética a sus padres pero también nos vamos asemejando cada vez más por la educación que recibimos durante toda la vida. A mi hermano, le agradezco la entereza, el ánimo y el cariño que nunca me faltó. Hay muchas formas de dar cariño y es mutuo. A mi cuña le agradezco como una hermana más su cariño y ayuda que siempre desde su madurez me brindo. A Juan (que nos falta) y Blanca les agradezco que me abrieran las puertas de su casa como si fuera uno más, ella sabe que mucho de este trabajo se ha realizado gracias a su ayuda.

Por suerte para mí, en mi vida se cruzó mi mujer, Azahara, curiosamente cuando comenzamos biología y también cuando conocimos a Justo. De hecho, ella es la única persona que ha conocido en presente todas y cada una de las situaciones que he vivido durante estos años. Tal vez, siempre recuerdas la ayuda que te han prestado en el laboratorio, tus directores, compañeros de trabajo, consejos de amigos,... Yo puedo decir que he recibido una ayuda inestimable e incalculable desde las sombras, de aquello que sólo muy pocos ven. Durante el transcurso de esta Tesis he vivido momentos muy amargos, algunas veces he querido tirar la toalla, sin embargo, siempre tuve un as en la manga, ella ha sido el elemento crucial que me ha permitido llevar a cabo esta Tesis.

A Azahara

A mis padres

A mi hermano

List of publications

This Thesis is based on the research articles listed bellow, which will be referred in the text by their Roman numerals.

I. **José Córdoba-Chacón**, Manuel D. Gahete, Mario Durán-Prado, Ana I. Pozo Salas, María M. Malagón, Francisco Gracia-Navarro, Rhonda D. Kineman, Raul M. Luque, and Justo P. Castaño. 2010. *Identification and characterization of new functional truncated variants of somatostatin receptor subtype 5 in rodents.* Cellular and Molecular Life Sciences. 67(7):1147-63.

II. **José Córdoba-Chacón**, Manuel D. Gahete, Justo P. Castaño, Rhonda D. Kineman, and Raul M. Luque. 2010. *Somatostatin and its receptors contribute, in a tissue-specific manner, to the sex-dependent, metabolic (fed/fasting) control of growth hormone axis in mice.* American Journal of Physiology: Endocrinology and Metabolism. In press. doi:10.1152/ajpendo.00514.2010.

III. **José Córdoba Chacón**, Rhonda D. Kineman, Justo P. Castaño, and Raúl M. Luque. *Cortistatin is not a somatostatin analogue but stimulates prolactin release and its deficit causes plasma insulin decrease and male-selective glucose impairment: role of ghrelin.* Manuscript.

IV. **José Córdoba Chacón**, Rhonda D. Kineman, Justo P. Castaño, and Raúl M. Luque. *Low doses of somatostatin signal through sst5 and AC/cAMP to dramatically increase GH release in primary pituitary cell cultures from a non-human primate (Papio anubis).* Manuscript.

V. **José Córdoba Chacón**, Rhonda D. Kineman, Justo P. Castaño, and Raúl M. Luque. *Homologous and heterologous in vitro regulation of pituitary receptors for somatostatin, growth hormone (GH)-releasing hormone and ghrelin in a non-human primate (Papio anubis).* Manuscript.

VI. Raul M. Luque, **José Córdoba-Chacón**, Manuel D. Gahete, Victor M. Navarro, Manuel Tena-Sempere, Rhonda D. Kineman, and Justo P. Castaño. 2010. *Kisspeptin regulates gonadotroph and somatotroph function in non-human primate pituitary via common and distinct signaling mechanisms. Endocrinology.* In press.

List of Abbreviations

aa – Amino acid
AC – Adenylate cyclase
ACTH – Adrenocorticotropic hormone
$[Ca^{2+}]_i$ – Intracellular calcium concentration
cAMP – Cyclic 3',5' adenosine monophosphate
cDNA – Complemetary deoxiribonucleic acid
CDS – Coding sequence
CNS – Central nervous system
CRF – Corticotropin releasing factor
CRF-R - Corticotropin releasing factor receptor
CST - Cortistatin
DNA – Deoxiribonucleic acid
E2 - Estradiol
FSH – Follicle stimulating hormone
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GAS – Glycoprotein α-subunit
GC – Guanylate cyclase
GH – Growth hormone
GHRH – Growth hormone releasing hormone
GHRH-R - Growth hormone releasing hormone receptor
GHS-R – Ghrelin receptor or Growth hormone secretagogue receptor
GIT – Gastrointestinal tract
GnRH – Gonadotropin releasing hormone
GnRH-R – GnRH receptor
GOAT – Ghrelin O-acyl transferase
GPCR – G protein coupled receptor
GTT – Glucose tolerance test
HPT - Hypothalamus
HPRT – Hypoxanthine guanine phosphoribosyltransferase
IGF-I – Insulin like growth factor type I
IGFI-R – IGF-I receptor
IP – Inositol phosphate
ITT – Insulin tolerance test
Kp – Kisspeptin
KO – Knockout
LH – Luteinizing hormone
L-NAME - N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride
MAPK - Mitogen-activated protein kinase
MEM – Minimun essential medium
mRNA – Messenger ribonucleic acid
mTOR – mammary target of rapamycin
MT-hGHRH - metallothionein promoter-driven, human growth hormone-releasing hormone
NOS – Nitric oxide synthase
NPY – Neuropeptide Y
PACAP - pituitary-adenylate cyclase-activating polypeptide
PBS – Phosphate buffer solution
PCR – Polimerase chain reaction
PI3K – Phosphatidylinositol 3 kinase
PIT - Pituitary
PKA – Protein kinase A
PKC – Protein kinase C
PLC – Phospholipase C
POMC – Propiomelanocortin
qrtRT-PCR – quantitative real time RT-PCR
PRL – Prolactin
PRLRH – Prolactin releasing hormone
PRLRH-R – PRLRH receptor
RNA – Ribonucleic acid
RT - Retrotranscription
RT-PCR – Retrotrancriptase-PCR
SEM – Standard error of the mean
SST - Somatostatin
Sst – somatostatin receptor
TMD – Transmembrane domain
TPA – 12-O-tetradecanoyl-phorbol-13-acetate
TRH – Thyrotropin releasing hormone
TSH – Thyroid stimulating hormone
Ucn - Urocortin
VIP – Vasoactive intestinal peptide

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Introduction

Regulation of growth hormone secretion

Growth hormone (GH; somatotropin) is a protein hormone secreted by the somatotropes of the anterior pituitary gland (adenohypophysis) (Schaufele, F 1994). GH secretion shows a pulsatile pattern in all species studied to date. In particular, humans and rodents exhibit a sexually dimorphic pattern of GH secretion. Specifically, GH secretion occurs in discrete pulses with low interpeak levels in male, whereas GH release displays lower pulsatility in females, where interpeak levels are higher (Bluet-Pajot, MT *et al.* 1998; Giustina, A *et al.* 1998). In the last four decades, major efforts have been done to elucidate the functions and regulation of GH. Its role in the control of somatic growth, as well as complex metabolic actions that greatly influence body composition have stimulated both human health-related and animal production-related research. Thus, GH is released into the general circulation where it interacts with multiple peripheral tissues through its receptor, GHR, to ultimately regulate growth and metabolic function. It has become increasingly clear that somatic growth is tightly interconnected with nutrition and reproductive status (Hull, KL *et al.* 2002; Maccario, M *et al.* 2000; Norrelund, H 2005). As a consequence, the biological actions of GH are not restricted to growth promotion; instead they include energy mobilization, body composition, gonadal development, etc. (Hull, KL *et al.* 2002; Norrelund, H 2005; Vijayakumar, A *et al.* 2010). Similarly, the regulatory network for GH is highly intricate, including many endocrine and environmental factors appropriate for the diverse physiological circumstances in which GH is involved.

Classically, regulation of GH secretion has been attributed to the "dual control" system played by two hypothalamic factors with opposing roles: GH releasing hormone (GHRH, which is mainly expressed in the arcuate nucleus of the hypothalamus, ARC) and somatotropin release inhibiting factor (somatostatin, SRIF or SST; which is mainly expressed in the periventricular nucleus of the hypothalamus, PeVN) (Bluet-Pajot, MT *et al.* 1998; Giustina, A *et al.* 1998; Tannenbaum, GS, Epelbaum, J 1999). Thus, it has been shown that GHRH and SST act in a reciprocal manner to maintain GH release in a well-defined range and their relative contribution is dependent on age, sex and metabolic status (Giustina, A *et al.* 1998; Hull, KL *et al.* 2002; Norrelund, H 2005). However, nowadays it is widely accepted that the episodic secretion of GH can be profoundly modulated by diverse factors residing in the target organ, the pituitary, other regions of the central nervous system, or by factors arriving from peripheral organs/tissues. For instance, ghrelin [which is mainly expressed in the gastrointestinal tract (GIT)] and

cortistatin (CST or CORT, which is predominantly expressed in the cerebral cortex) have been shown to operate as two important factors able to stimulate and inhibit, respectively, GH release by acting directly at somatotrope cells via their respective GH-secretagogue receptor (GHS-R) and somatostatin-receptors (ssts) (Broglio, F *et al.* 2002a; de Lecea, L *et al.* 1996; Dimaraki, EV *et al.* 2006; Korbonits, M *et al.* 2004; Rubinfeld, H *et al.* 2006). Also, pituitary-adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP)/GHRH family primarily produced in the hypothalamus, can stimulates GH release and expression in a variety of species (from fish to mammals) however, in mammals the actions of PACAP seems to be weaker than those of GHRH (Malagon, MM *et al.* 2003). In addition, Kisspeptins (Kps; the peptide products of the Kiss1 gene, which bind to specific receptors known as Kiss1r/GPR54) have recently emerged as new putative stimulatory modulators of GH secretion (Ezzat Ahmed, A *et al.* 2009; Gutierrez-Pascual, E *et al.* 2007; Kadokawa, H *et al.* 2008a; Ramaswamy, S *et al.* 2009). In fact, there is emerging evidence indicating that all these peptides (SST/CST/ghrelin/GHRH/kp) and receptors comprise a network of physiologically interrelated components able to functionally interact at the molecular and cellular level to regulate multiple pituitary cell-axes (including somatotrope function) under normal and pathological conditions. However, the precise contribution, regulation and components of some of these actions are still uncertain or controversial and, as will be discussed later, cannot be explained by the interaction with their known specific receptors. Accordingly, there are still a number of questions that remain to be solved in order to fully understand the (patho)-physiological functions that have been associated to these family of peptides (for instance, the importance of endogenous CST in maintaining GH-axis function in vivo). Accordingly, the initial set of studies of the present research work have been mainly focused at determining the exact role that endogenous SST/CST/receptors play in regulating GH output under basal and/or altered metabolic conditions (i.e. fasting) and to ascertain their interaction with the ghrelin/GHSR system using two models [*Mus musculus* (male and female mice) and *Papio anubis* (female baboons)]. Additional studies were also performed in order to determine, for the first time, the direct pituitary actions of Kps on pituitary hormone release using the female baboon as a model.

Somatostatin and Cortistatin system: background

SST and CST are two cyclic neuropeptides which share remarkable sequence, structural and functional similarities, as they seem to evolve from a common ancestral gene (Gahete, MD *et al.* 2010a; Tostivint, H *et al.* 2004; Tostivint, H *et al.* 2006; Tostivint, H *et al.* 2008). In spite of their high homology, the discovery of SST preceded that of CST by more than two decades. SST was discovered in 1973 in ovine hypothalamus by its ability to inhibit GH secretion, thus its name (Brazeau, P *et al.* 1973). In contrast, CST was initially identified in 1996, almost simultaneously in rodents (de Lecea, L *et al.* 1996) and amphibians (Tostivint, H *et al.* 1996) and immediately thereafter in humans (Fukusumi, S *et al.* 1997), and named cortistatin by its high expression in the cerebral cortex and its ability to depress neuronal electrical activity. In addition, SST and CST preproproteins have similar post-translational maturation, which gives rise to distinct products after enzymatic cleavage, SST-14 and SST-28 from preproSST and their analogous CST peptides CST-17 (humans)/CST-14 (rodents) and CST-29 (humans and rodents) from preproCST [for review, see (Gahete, MD *et al.* 2010a; Gahete, MD *et al.* 2008; Spier, AD *et al.* 2000)]. Nonetheless, SST and CST mature products share two elements that are essential for their similar receptor binding and functional abilities, a disulfide bridge to stabilize their cyclic structure and a FWKT domain. The complexity and versatility of the SST and CST systems are also influenced by their receptors. In fact, SST and CST bind with similar subnanomolar affinity to a group of guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCR) named somatostatin receptors (sst) (Moller, LN *et al.* 2003). Specifically, five different genes have been identified to date, which encode five different receptor subtypes (sst1-5). Moreover, it has been demonstrated that post-transcriptional events generate additional spliced receptors by either canonical splicing from sst2 (sst2B) in various species (Vanetti, M *et al.* 1992) or by non-canonical splicing from sst5 in humans (sst5TMD5 and sst5TMD4) (Duran-Prado, M *et al.* 2009) and pigs (sst5TMD6 and sst5TMD3) (Duran-Prado, M 2007). Therefore, contrary to the earlier "one hormone-one receptor-one function" simplistic and unidirectional notion, it is now well established that SST/CST system is in fact composed of multiple peptides and various receptors (long-forms and spliced variants) which can act in concert to exert a number of actions.

Somatostatin and Cortistatin system: functions

SST, originally identified by its ability to inhibit the secretion of GH from pituitary somatotropes (Ben-Shlomo, A *et al.* 2010; Patel, YC 1999), is nowadays recognized as a multifarious, widespread peptide able to regulate a number of (patho)-physiologically relevant functions as distinct as neurotransmission (Viollet, C *et al.* 2008), locomotor and cognitive function (Haroutunian, V *et al.* 1987), vascular contractility (Guillermet-Guibert, J *et al.* 2005), insulin release, glucose homeostasis (Gerich, JE 1981), or gastrointestinal motility (Van Op den Bosch, J *et al.* 2009). On the other hand, the precise functional roles of CST are still less well understood. The limited data available so far regarding its endocrine activities indicate that CST, like SST, also inhibits GH secretion and influences similar endocrine and non-endocrine processes (Broglio, F *et al.* 2008; Moller, LN *et al.* 2003; Olias, G *et al.* 2004; Patel, YC 1999). However, to date, no studies have thoroughly investigated the precise actions and potential physiological relevance of CST at the pituitary level and its metabolic interface, despite evidence suggesting that SST and CST are more than two "endocrine siblings" (Gahete, MD *et al.* 2008).

Since its discovery, knowledge on the mechanisms underlying the ability of SST to inhibit GH secretion by pituitary somatotropes has continuously been growing, and recent studies have precisely delineated that this inhibitory action is mainly exerted via receptors sst2 and sst5 (Ben-Shlomo, A *et al.* 2010; Patel, YC 1999). Although the expression of CST in hypothalamus extract is significantly lower as compared to SST (Allia, E *et al.* 2005; de Lecea, L *et al.* 1996; Luque, RM *et al.* 2007a), CST has also been shown to be able to inhibit GH secretion (Baranowska, B *et al.* 2006; Broglio, F *et al.* 2002a; Luque, RM *et al.* 2006e; Rubinfeld, H *et al.* 2006). However, it should be mentioned that the understanding of the direct effects of CST in regulating the secretion of pituitary hormone is somewhat controversial and incomplete, and has largely been based on studies conducted in non-primate species or in pituitary cell cultures established from human fetal pituitaries or GH-producing adenomas. As we will describe below (Section of "Somatostatin and Cortistatin system: Regulation of pituitary physiology"), the effects of SST on the release of pituitary hormones are not exclusive restricted to somatotropes, as it has been demonstrated that SST can also inhibit the secretion of other pituitary cell types, such as lactotropes, corticotropes, thyrotropes and gonadotropes; however, once again, the potential inhibitory effect of CST on the

secretion of all these pituitary cell types has not been reported hitherto [for review, see (Gahete, MD *et al.* 2008)].

Besides its pituitary actions, it is well known that SST can inhibit hypothalamic release of GH-releasing hormone (GHRH) in ARC and SST secretion from somatostinergic neurons of PeVN through binding both sst1 and sst2 (Bluet-Pajot, MT *et al.* 1998; Viollet, C *et al.* 2008). Moreover, in central nervous system, SST can act as a neuromodulatory agent of glutamatergic or GABAergic transmission or as a neurotransmitter by itself (activating K^+ channels and inhibiting Ca^{2+} influx) (Viollet, C *et al.* 2008). On the other hand, CST actions go from inhibition of glutamatergic transmission in hippocampus with the subsequent impairment of long-term potentiation, to enhancement of slow-wave sleep through hyperpolarization of cortical neurons which antagonizes with excitatory effects of acetylcholine and enhanced-REM sleep induced by SST (de Lecea, L 2008; de Lecea, L *et al.* 1996). In addition, SST and CST have effects in locomotor activity, which is increased by SST and decreased by CST (de Lecea, L *et al.* 1996; Haroutunian, V *et al.* 1987).

Interestingly, SST and CST have also been shown to be involved in the modulation of the immune system. Indeed, although CST (but not SST) is expressed in cells of the immune system (Dalm, VA *et al.* 2003), SST exerts inhibitory effects on monocytes, B- and T-cells (van Hagen, PM *et al.* 2008) while CST exhibits an inhibitory effect on production of pro-inflammatory signals and a stimulatory action on expression of anti-inflammatory cytokines (Gonzalez-Rey, E *et al.* 2008). Moreover, SST is highly expressed throughout the GIT, the liver and endocrine pancreas (Dalm, VA *et al.* 2004) and it causes suppressive effects on gastric emptying, gastric secretion, gallbladder contractility, propulsive activity of the small and large intestines, pancreatic secretion of glucagon and insulin, regulation of bile formation, and inhibition of cytokine release by liver stellate cells (Van Op den Bosch, J *et al.* 2009). In this same context, and although at lower levels than SST, CST is also present in GIT (Dalm, VA *et al.* 2004); however, to date the specific roles of CST in the digestive system have not been described in detail, despite some results revealing CST could have SST-like actions, at least at stomach level (Broglio, F *et al.* 2002b).

The overlapping functions described hitherto for SST and CST can be explained by their close pharmacology (Siehler, S *et al.* 2008), as both peptides exhibit similar

subnanomolar binding affinity to the five sst subtypes, which, in turn, exhibit a widespread co-expression throughout the organism (Dalm, VA *et al.* 2004; Patel, YC 1999). However, unique specific actions for SST or CST might be triggered by the absence of one of these two ligands in a specific cell/tissue target or by differential modulation of signaling/physiology of the receptors, for instance via homo- and/or hetero-dimerization events (Duran-Prado, M *et al.* 2008). Furthermore, it has been described that CST, but not SST, can bind to non-sst GPCRs, such as the ghrelin receptor type-1a (GHS-R1a) (Deghenghi, R *et al.* 2001b) and the Mas-related G-protein coupled receptor X2 (MrgX2; a specific receptor from the primate lineage) (Burstein, ES *et al.* 2006; Robas, N *et al.* 2003). However, although CST could binds to these receptors *in vitro*, a possible physiological role *in vivo* has not been clarified as yet (Nothacker, HP *et al.* 2005; Sibilia, V *et al.* 2006; Siehler, S *et al.* 2008). Notwithstanding, it has also been postulated that additional specific receptors related to sst1-5 may exist that would trigger specific and selective actions of SST or CST (de Lecea, L *et al.* 2006; Deghenghi, R *et al.* 2001b; Robas, N *et al.* 2003), as it is the case of the truncated variants of sst5 described by our group in humans and pig which can exhibit selective responses to SST or CST *in vitro* (Duran-Prado, M 2007; Duran-Prado, M *et al.* 2009; Durán-Prado, M *et al.* 2005).

Ghrelin and Somatostatin/Cortistatin system: Ghrelin is a 28 amino acid peptide that was originally identified by Kangawa's group from a gastric extract (Kojima, M *et al.* 1999). Ghrelin can be acetylated in its third aminoacid (Serine) with an octanoyl group by ghrelin o-acyl transferase (MBOAT or GOAT) (Gutierrez, JA *et al.* 2008) which give rise the active ghrelin with binding capacity to ghrelin receptor GHS-R1a. Of note, no other naturally occurring peptide has been previously shown to have this acyl-group as a posttranslational modification. There is emerging evidences to support the notion that, rather than being isolated neuropeptide-receptor systems with distinct, separate functions, SST, CST, ghrelin and their related peptides and receptors comprise a network of physiologically interrelated components able to functionally interact at the molecular, cellular and organismal level to regulate multiple functions in health and disease. For instance, as pointed out earlier, ghrelin is well known for being an additional regulatory factor of somatotrope cells (Gahete, MD *et al.* 2009), and despite exerting opposite actions on GH release than SST/CST (stimulatory *vs.* inhibitory) it shares many features with these peptides. Thus, like SST and CST, ghrelin is generated from a pre-proprotein to produce several peptides, including diverse ghrelin variants and obestatin (Seim, I *et al.* 2009). Also, similar to SST, ghrelin is mainly produced in stomach and regulates GIT

physiology, but it is also present in the hypothalamus, where it plays hypophysiotropic actions and exhibits important roles in the regulatory interplay between endocrine and metabolic processes (Cordido, F *et al.* 2009; Chen, CY *et al.* 2009). Moreover, it has been found that SST infusion reduces plasma ghrelin levels in rats (Shimada, M *et al.* 2003), and humans (Norrelund, H *et al.* 2002). On the other hand, ghrelin seems to exert a divergent action on SST release at systemic and local levels since ghrelin infusion seems to elevate plasma SST levels in humans (Arosio, M *et al.* 2003) while it reduces SST secretion in pancreas explants (Egido, EM *et al.* 2002). An additional line of evidence supporting the close relationship between SST/CST and ghrelin systems, which also increases the complexity of these networks comes from the recent demonstration that sst5 can physically interact with GHS-R1a forming heterodimers (Jiang, H *et al.* 2007), which could modify the signaling elicited by ghrelin and/or SST. However, the functional consequences of this interaction are still unknown. Altogether, it seems reasonable to propose that SST/CST, ghrelin and their receptors and related peptides comprise two highly interrelated pleiotropic systems with yet unknown functions, and therefore, that whenever possible these systems should be studied as a whole in future studies.

SST/CST/sst1-5 system: mechanism of signaling

As mentioned above, much of the functional versatility of SST, and likely of CST, is likely related to their family of widely distributed sst receptors (Moller, LN *et al.* 2003; Patel, YC 1999). ssts are often present simultaneously in the same cells, where they functionally interact with each other or with other GPCR forming homo- and/or hetero-dimers to activate different signaling cascades and mediate multiple actions (Duran-Prado, M *et al.* 2008; Somvanshi, RK *et al.* 2009; Watt, HL *et al.* 2009). Therefore, the classic conception of this regulatory system composed by several ligands/receptors (long-forms and spliced variants) joined to a simple intracellular signaling pathway is not enough to explain the wide variety of functions of SST/CST/sst1-5. Hence, a multi-factorial intracellular signaling complex is coupled to the distinct G proteins and GPCR-interacting proteins to trigger the SST/CST signaling into the cell. In fact, sst1-5 can act through more than 20 intracellular signaling pathways to develop SST/CST actions [for review, (Cervia, D *et al.* 2007), see adapted Table 1]. Therefore, for the sake of clarity and space limitations, we will describe the germane specific intracellular signaling pathways of each receptor in the corresponding section of this Thesis.

Table 1. Intracellular signaling pathways triggered by SST or specific sst1-5 in native systems (Table adapted from Cervia D et al. 2007 Pharmacology & Therapeutics 116:322–341.)

	Contribution of SST or specific sst1-5					
Transduction pathways	**SST**	**sst1**	**sst2**	**sst3**	**sst4**	**sst5**
Adenylyl cyclase	↓↑	↓	↓	↓↑	↓	↓↑
Guanylyl cyclase	↑↓		↑			
Nitric oxide	↓↑	↑(?)	(?)↓↑	↓		↓
Phospholipase C	↓↑		↑	↑		↑
$[Ca^{2+}]_i$	↓↑	↓	↓↑	↑		↓↑
K^+ conductances	↑		↑			
Inwardly rectifying K^+ channels	↑	↓	↑			↑
Delayed rectifying K^+ channels	↓↑	↑	↑		↑	↑
Transient outward K^+ channels	↑	↑		↑	↑	
Ca^{2+}-activated K^+ channels	↓↑		↓↑			
ATP-sensitive K^+ channels	↓↑					↑
Phospholipase A2	↓↑	↓	↓			
Na^+/H^+ exchanger	↓↑	(?)↓	(?)↓			(?)↓
Protein-tyrosine phosphatases	↑		↑			↑(?)
SHP-1	↑		↑			↑(?)
SHP-2	↑	↑				
r-PTPη	↑					
DEP-1/PTPη	↑					
Protein serine/threonine phosphatases	↑					
PP2A	↑(?)			↑(?)		
PP2B	↑					
MAP kinase	↓↑	↓↑	↓↑	↓↑(?)	(?)↓	↓
PI3 kinase	↓↑	↑(?)	↓↑(?)	↑		(?)↓↑(?)
PDK1	↓					
Akt	↓	↑(?)	(?)↓↑(?)			(?)↓↑(?)
GSK3β	↑		↓			
c-src	↑		↑			
cdk	↓		↓			(?)↓
$P70^{S6}$ kinase	↓					
IKK			↑			
Janus kinase			↑			
ILK				↑		
CaMKII						↑(?)
Small G proteins	↓					
GRK2	↑					

(?)Involvement of the specific receptor needs further demonstration.

Somatostatin and Cortistatin system: Regulation of pituitary physiology

As briefly outlined above, SST, and to a lesser extent CST, act as inhibitory peptides for various endocrine secretions, being their best well-known target tissue the cell populations of the pituitary gland (Broglio, F *et al.* 2008; Gahete, MD *et al.* 2008). Pituitary gland or hypophysis is an endocrine gland located at the "sella turcica" or

pituitary fossa in the sphenoid bone at the base of the brain. The mammalian pituitary is constituted by two distinct lobes: 1) Adenohypophysis or anterior pituitary which is developed from Rathke's pouch, an embryonic structure that arises from oral ectoderm and, 2) Neurohypophysis or posterior pituitary which is generated from neural ectoderm from the base of the developing diencephalon. Neurohypophysis is directly connected with hypothalamus through the infundibular stalk, where axons of hypothalamic neurons pass from median eminence to this structure. In addition, neurons from different hypothalamic nuclei release regulatory factors to the hypothalamic-hypophyseal portal veins located in the infundibular stalk which branch again into another series of capillaries to establish a direct functional interplay between hypothalamic nuclei and endocrine cells within the adenohypophysis (Bargmann, W 1981). Five types of endocrine cells are present in the adenohypophysis: somatotropes, lactotropes, gonadotropes, corticotropes, and thryrotropes, which, produce and release six hormones that could be classified in order to their molecular features (polypeptides and glycoprotein hormones). On the one hand, growth hormone (GH), adrenocorticotropin hormone (ACTH) and prolactin (PRL) are polypeptide hormones produced by somatotropes, corticotropes and lactotropes, respectively. On the other hand, thyroid-stimulating hormone beta-subunit (TSH-β) produced by thyrotropes and follicle-stimulating hormone beta-subunit (FSH-β) and luteinizing hormone beta-subunit (LH-β) produced by gonadotrope cells are glycoprotein hormones which dimerize with a common glycoprotein alfa-subunit (GAS) produced in these cells to form the active hormones (Bargmann, W 1981; Horvath, E *et al.* 1994). Because the requirement of pituitary hormones depends on the metabolic/physiologic needs of the organism and target cells, their synthesis and release are under a fine-tuning regulation, which has been shown to be sex-dependent and present circadian rhythm oscillations related to light/dark, sleep/wake cycles, etc (Gan, EH *et al.* 2010). It should be noted that the final concentration of these hormones into the bloodstream is defined by a precise interplay between releasing and inhibiting factors arriving not only from different hypothalamic nuclei but also from other, central and systemic, endocrine tissues (i.e. stomach, liver, pancreas, etc) (Barb, CR *et al.* 2002; Gahete, MD *et al.* 2009; Luque, RM *et al.* 2011). In sum, pituitary is a neuroendocrine organ comprised by two different anatomical regions (neuronal and epithelial) with the capacity to release multiple hormones that regulate many functions throughout the organism.

A) Regulation of GH biosynthesis and secretion: Overview and SST and CST role.

The secretion of GH by somatotrope is tightly regulated by positive and negative signals released from pituitary, hypothalamus, stomach, adrenals, adipose tissue, gonads, liver, and pancreas. Integration of these signals by somatotrope cells results in daily peaks in the secretion of GH, which characterize its typical intradian/circadian variations. Moreover, sex steroids mediate gender-dependent variations in the levels of plasma GH. Thus, as mentioned above, males tend to have a GH secretion pattern highly organized, with high amplitude pulses and low baseline values female pattern is more disorganized, with increased pulse frequency and elevated baseline values (Giustina, A *et al.* 1998). Although classic models designed to explain this patterns of GH synthesis and release conceived it basically as a "dual control" system exerted by a stimulatory factor, GHRH, and an inhibitory factor, SST (Bluet-Pajot, MT *et al.* 1998; Giustina, A *et al.* 1998), it is nowadays accepted that other central and systemic signals can also profoundly influence and/or alter this dual-hormone driven pattern, both positively (i.e. ghrelin, kp, thyroid hormones, PACAP, glucocorticoids and leptin) and negatively [i.e. CST, insulin, insulin-like growth factor type-I (IGF-I), free fatty acids (FFA)], regulate GH synthesis and/or secretion (Gahete, MD *et al.* 2009; Giustina, A *et al.* 1998; Luque, RM *et al.* 2011).

In line with this notion, GH synthesis and release is directly and profoundly regulated under extreme metabolic conditions, since nutrient deficiency (i.e. fasting, anorexia, uncontrolled diabetes type I) results in an increase of plasma GH concentration (Mercado, M *et al.* 1995; Norrelund, H 2005; Scacchi, M *et al.* 2003) while, nutrient excess and the subsequent obesity induces GH suppression (Maccario, M *et al.* 2000; Scacchi, M *et al.* 1999). Indeed, nutrient deficiency produces an elevation of interpulse basal level, peak frequency and peak height of GH release, which could be due, at least in part, to the fact that the tones of GHRH, acyl-ghrelin (active form of ghrelin that binds to and activates GHSR-1a) and glucocorticoids are increased, whereas those of SST, IGF-I and insulin are decreased under this metabolic condition, with all these changes favoring an increase in GH output. Furthermore, under these conditions, pituitary cells are more sensitive to the stimulatory effect of GHRH and ghrelin and less sensitive to the inhibitory effect of SST (Luque, RM *et al.* 2009 ; Luque, RM *et al.* 2007b; Luque, RM *et al.* 2008; Mercado, M *et al.* 1995; Norrelund, H 2005; Scacchi, M *et al.* 2003; Zhao, TJ *et al.* 2010). On the other hand, mechanisms by which nutrient excess reduce plasma GH levels are poorly understood. However, it has been suggested that the high levels of circulating insulin, IGF-I, leptin, glucose and/or FFA as well as the low levels of ghrelin observed in obesity could be, in part, responsible of the suppression of the GH-axis observed in this

metabolic state (Cattaneo, L *et al.* 1996; Luque, RM *et al.* 2011; Luque, RM *et al.* 2006d; Luque, RM *et al.* 2009 ; Luque, RM *et al.* 2008). Moreover, it should be noted that nutrient excess-dependent GH suppression has been associated with defects in hypothalamic input (suppressed GHRH and enhanced SST tone). However, there are also evidences implicating defects in somatotrope function (i.e. decreased GH, GHRH-R and GHS-R) independent of changes in hypothalamic GHRH and SST expression (Cattaneo, L *et al.* 1996; Luque, RM *et al.* 2006d; Luque, RM *et al.* 2009 ; Luque, RM *et al.* 2008).

Interestingly, our research group has reported the existence of a paradoxical, dual inhibitory/stimulatory effect of SST on GH release exerted directly upon primary pituitary cell cultures from pigs (Castaño, JP *et al.* 2005; Castaño, JP *et al.* 1996; Luque, RM *et al.* 2006e; Ramirez, JL *et al.* 1998; Ramirez, JL *et al.* 1997). Indeed, although it has been demonstrated that SST is the major inhibitory factor of basal and stimulated GH release when applied at high concentrations, our laboratory have also reported that low doses (subnanomolar to picomolar range) of SST can produce a potent increase of GH release (Castaño, JP *et al.* 2005; Castaño, JP *et al.* 1996; Luque, RM *et al.* 2006e; Ramirez, JL *et al.* 1998; Ramirez, JL *et al.* 1997). This stimulatory effect induced by low doses SST in porcine pituitary cells was mediated through sst5 (Luque, RM *et al.* 2006a) and by an increase of intracellular cAMP levels (Ramirez, JL *et al.* 2002). Moreover, further studies demonstrated also that high and low doses of SST differentially regulate the expression of pituitary receptors involved in the regulation of GH release (sst1-5, GHRH-R and GHS-R) (Luque, RM *et al.* 2004a; Luque, RM *et al.* 2004b). Interestingly, it should be noted that CST seems to mimic the stimulatory effect of SST on GH release in porcine (Luque, RM *et al.* 2006e) and rat (Baranowska, B *et al.* 2006) primary pituitary cells cultures.

B) Regulation of ACTH biosynthesis and secretion: Overview and SST and CST role.

Pituitary corticotrope cells produce proopiomelanocortin (POMC), a preprohormone which is cleaved by prohormone convertase 1 (PC-1) to produce mature adenocorticotropin (ACTH) (Low, MJ *et al.* 1993; Mizuno, K *et al.* 1994). Expression of POMC and secretion of ACTH is regulated by hypothalamic corticotropin-releasing factor (CRF) through binding to CRF receptor type-1 (CRF-R1) which is highly expressed in corticotropes (Sakai, K *et al.* 1996; Van Pett, K *et al.* 2000). However, other hypothalamic signals could also act as positive inputs on ACTH secretion (i.e. vasopressin and oxytocin acting through vasopressin 1b receptor (V1bR) and urocortins

through CRF-receptors) (Fekete, EM *et al.* 2007; Ur, E *et al.* 1994). Once in the bloodstream, the main role of ACTH is to stimulate adrenocortical steroidogenesis and secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) through binding to melanocortin receptor 2 in the adrenal cortex (Chida, D *et al.* 2007; Mountjoy, KG *et al.* 1992). Glucocorticoids are considered the main inhibitory signal of ACTH release acting directly at the pituitary level, or indirectly at brain sites (i.e. hypothalamus and hippocampus) through binding to glucocortioid or minerolocorticoid receptors expressed in those target tissues (Funder, JW 1996). In line with this idea, the CA1 region of the hippocampus seems to be the main area mediating the inhibitory feedback of ACTH release. In fact, it has been indicated that glucocorticoids bind mineralocorticoid receptor-expressing neurons of the hippocampus which project to inhibitory GABAergic neurons of the peri-paraventricular nucleus (PVN) that impinge on CRF neurons in the hypothalamus (Jacobson, L 2005; Ziegler, DR *et al.* 2002). Interestingly, it has been shown that ACTH secretion is also under a circadian rhythm controlled by the levels of ACTH itself as well as by glucocorticoids, CRF, vasopressin and oxytocin levels, by the light/dark cycle through suprachiasmatic nucleus of the hypohalamus and by food intake (Jacobson, L 2005; Ur, E *et al.* 1994; Ziegler, DR *et al.* 2002). In addition, it has been reported that SST and CST (Giordano, R *et al.* 2007; Hofland, LJ *et al.* 2005) could also suppress basal and CRF-stimulated ACTH release, and this effect, which particularly relevant in Cushing's disease (pituitary-dependent ACTH-driven glucorticoid hypersecretion) seems to be mediated preferentially via sst5 and not through sst2, since elevated glucocorticoid levels caused by ACTH hypersecretion selectively inhibit sst2 expression. Moreover, in support of a close relationship between the corticotropic and SST/CST systems, it is worth noting lack of endogenous SST in mice produces an increase in POMC expression and corticosterone levels (Luque, RM *et al.* 2006b), and also, that other systemic signals such us cytokines can stimulate the hypothalamus-pituitary-adrenal axis HPA and the release of cytokines, in turn, can be directly regulated by the SST/CST system (Gonzalez-Rey, E *et al.* 2008).

C) Regulation of PRL biosynthesis and secretion: Overview and SST and CST role.

Synthesis and release of prolactin by lactotropes is mainly regulated by factors produced in the hypothalamus, as well as, in the pituitary itself, gonads, and liver. Of these factors, it has been extensively described that dopamine, a neurotransmitter produced by several hypothalamic regions, exerts a primary inhibitory role on lactotrope function, as it inhibits their PRL synthesis and secretion mainly through dopamine

receptor type-2 (DR2) (Ben-Jonathan, N *et al.* 2001). In addition, estrogens produced by gonads represent a major stimulatory input increasing prolactin expression and release, by acting directly, via binding to estrogen receptor (ER)-alpha and ER-beta in the pituitary, also indirectly, by inhibiting dopamine release in the hypothalamus via ER-alpha (Freeman, ME *et al.* 2000; Mitchner, NA *et al.* 1998). Moreover, estrogens induce proliferation of lactotropes, this likely underlying the characteristic gender difference of the prolactin-axis in both lactotrope cell number and prolactin levels, which are markedly higher in females (Ben-Jonathan, N *et al.* 2001; Freeman, ME *et al.* 2000). However, it should also be noted that the stimulatory role of estrogen in lactotrope physiology is not always associated with an effect on PRL secretion, since it has been indicated that some situations estrogen levels do not correlate with prolactin release (i.e. it has not been observed changes in PRL release in postmenopausal women taking antiestrogens) (Lasco, A *et al.* 2002). An additional factor that appears to relevantly contribute to regulate lactotrope function is IGF-I, since there is evidence that this hepatic factor is required for optimal expansion of the lactotrope population and can promote proliferation of lactotropes in primary rat pituitary cultures (Bartke, A 1999; Oomizu, S *et al.* 1998).

The quest for the primary prolactin releasing factor began more than 40 years ago, but has been completely unsuccessful since the stimulatory factors described to date mediate their actions mainly by the inhibition of dopamine (opioids) or by unknown indirect mechanisms (Andrews, ZB *et al.* 2003; Soaje, M *et al.* 2004). Thus, it has been reported that some hypothalamic (i.e. thyrotropin-releasing hormone, oxytocin) or pituitary (i.e. VIP) factors could exert a stimulatory action on prolactin release. Interestingly, in 1998 Hinuma *et al* (Hinuma, S *et al.* 1998) identified a candidate peptide, the prolactin releasing peptide (PrRP), from the bovine hypothalamus that was considered to serve as a hypothalamic releasing factor and to act on the anterior pituitary to stimulated PRL release; however, no PrRP inmunoreactivity was found in the external layer of the median eminence, from where classic hypothalamic hormones are released into portal blood to control anterior pituitary hormone release. Indeed, nowadays PrRP is not considered a classis hypothalamic hormone in mammals and instead, it appears to play a role in the control of energy metabolism and stress (Ben-Jonathan, N *et al.* 2008; Takayanagi, Y *et al.* 2010). Finally, it has been shown that SST and CST, mainly through sst5, can inhibit PRL release in fetal human pituitary cell cultures as well as in cultured prolactinomas (Grottoli, S *et al.* 2006; Rubinfeld, H *et al.*

2006). However, although SST and CST were able to inhibit PRL secretion in prolactinoma patients, these peptides did not alter PRL release in normal subjects (Broglio, F *et al.* 2008; Grottoli, S *et al.* 2006). Data from our laboratory and others indicate that SST can inhibit basal PRL release from female baboon pituitary cell cultures (Kineman, RD *et al.* 2007b) as well as TRH- or VIP-stimulated PRL secretion from cultured hemipituitaries from male rats (Enjalbert, A *et al.* 1982). Surprisingly, it has been reported recently that CST, but not SST, can stimulated the secretion of PRL in male rats in vivo (Baranowska, B *et al.* 2009). All together, there not seem to be a clear, unequivocal picture of the roles of SST and CST on the regulation of PRL secretion in normal subjects, and there are still unsolved questions of whether CST is a mere natural endocrine analogue of SST, or whether it possesses its own, physiologically relevant regulatory actions on pituitary secretions.

D) Regulation of TSH biosynthesis and secretion: Overview and SST and CST role.

As other pituitary hormones, TSH is secreted under a circadian rhythm controlled by several signals (Jackson, IMD 1994). Specifically, TSH release is controlled by complex mechanisms of positive and negative regulation that mainly involve two signals, one stimulatory, thyrotropin-releasing hormone (TRH), which is produced in the PeVN, and the inhibitory, the feedback exerted by thyroid hormones at the hypothalamic and pituitary levels (Jackson, IMD 1994). Circulating TSH affects thyroid-function by increasing the secretion of thyroid hormones (T_3 and T_4) which play critical roles in development, growth, and cellular metabolism (Chiamolera, MI *et al.* 2009). Moreover, it has been shown that other factors (such as SST, dopamine and glucocorticoids) could also regulate the hypothalamic-pituitary-thyroid axis in order to inhibit pituitary TSH release (Chiamolera, MI *et al.* 2009; Jackson, IMD 1994). Moreover, SST has been reported to inhibit stimulated thyrotropin release in vitro from fetal human pituitary cells (Shimon, I *et al.* 1997a), as well as in cultures of pituitary cells from rats and birds (Dieguez, C *et al.* 1984; Geris, KL *et al.* 2003).

E) Regulation of FSH and LH biosynthesis and secretion: Overview and SST and CST role.

The major regulator of synthesis and secretion of gonadotropins (FSH and LH) by pituitary gonadotropes is the gonadotropin-releasing hormone (GnRH) which is produced by a population of hypothalamic neurons and released in a pulsatile manner which in turn, modulates the release of FSH and LH also in a pulsatile fashion (Krsmanovic, LZ *et al.* 2009). Physiological effects of gonadotropins are focused on

gonads where they regulate many functions related with reproductive system. In response to gonadotropins, gonads produce steroid hormones (estrogen, testosterone, androgens, etc) that mediate an inhibitory feedback regulation at the hypothalamic level (GnRH neurons) and at the pituitary gonadotropes, controlling the synthesis and release of their products. Moreover, additional signals can promote stimulatory (i.e. kp, NPY, estradiol and norepinephrine) and/or inhibitory (beta-endorphin, progesterone, estradiol and interleukin-1) actions in GnRH neurons to facilitate the control of the pulsatile GnRH secretion and the subsequent release of gonadotropins (Kalra, SP *et al.* 1994; Krsmanovic, LZ *et al.* 2009; Tsutsumi, R *et al.* 2009). In line with this idea, it should be mentioned that the kps, the products of the Kiss1 gene that operate through the G protein-coupled receptor, Kiss1r (also known as GPR54), have taken central stage in Neuroendocrinology and Reproductive Physiology in the last five years. Specifically, it is well known that Kps act primarily at the hypothalamus to directly (or eventually indirectly) stimulate GnRH release, which would modulate FSH and LH release (Krsmanovic, LZ *et al.* 2009; Roseweir, AK *et al.* 2009). However, some evidences indicate that Kps could also exert direct modulatory effects on gonadotropin secretion at the pituitary level in that: 1) Kp is present in ovine hypophysial portal blood, suggesting that the PIT gland may be a target of Kp, 2) Kp/Kiss1r are highly expressed in the pituitary of several species; and 3) Kp can stimulate LH secretion in primary pituitary cultures from several non-primate species (mouse, rat, pig, cow, goldfish) (Richard, N *et al.* 2009; Roa, J *et al.* 2008). Accordingly, it has been suggested that Kp might act as endocrine/autocrine/paracrine signals in modulating gonadotropin in the pituitary; although, it is not clear that the actions of Kp observed in animal models, extends to humans. Finally, SST/CST system seems to have no major effect in the regulation of gonadotropin release, however, under pathological (human pituitary adenomas), but not normal conditions, SST appear to have a partial inhibitory effect on FSH or LH secretion (Gahete, MD *et al.* 2008). Therefore, some studies are necessary in order to elucidate the role of SST and CST on the gonadotropin-axis.

Somatostatin and Cortistatin knock-out mouse models.

The vast majority of the studies that have shed light on the regulation of pituitary hormone secretion have been developed using rodents as model. Because of their easy experimental manipulation, laboratory rodents (mainly, mice and rats) have been widely used to study the consequences that a patho-physiologic state could cause on specific tissue and cell functions. Likewise, rodents, especially mice, are ideally suited to study

the patho-physiological importance of gene products because of the feasibility to generate genetically modified mice over- or under-expressing the product of interest. Consequently, SST, CST or specific sst subtype knock-out mouse models have been developed by different groups to ascertain the precise role of these genes in the physiology of pituitary and other target tissues of SST/CST (Zeyda, T *et al.* 2008). Specifically, two distinct strains of SST knock-out models have been reported (Low, MJ *et al.* 2001; Zeyda, T *et al.* 2001) whereas to date, only one model of CST deficiency has been generated by Dr. de Lecea's group (de Lecea, L *et al.* 2010; Susuki, C *et al.* 2004; Tallent, MK *et al.* 2002). Scarce results of CST-KO model has been reported because its generation is recent and overall results are focused on neuronal physiology. However, SST-KO model has been more characterized at endocrine level, showing that absence of endogenous SST allows an increase of circulating GH (Luque, RM *et al.* 2007a; Zeyda, T *et al.* 2001) and ACTH levels with subsequent elevation of circulating corticosterone (Luque, RM *et al.* 2006b; Zeyda, T *et al.* 2001). Notwithstanding, these models lack one of the endogenous ligands for the sst1-5 and consequently, the other ligand (SST or CST) could bind to sst1-5 and compensate the lack of the other peptide. However, and as mentioned above, very little information is available about the lack of endogenous CST on endocrine/metabolic endpoints as compared with lack of endogenous SST, and therefore, it is clear that further studies are required to determine the impact of lack of sst1-5 signaling by SST and/or CST.

Somatostatin knock-out: Corticotroph and Somatotroph axes.

A partial endocrine characterization of SST-KO mice has been reported at the pituitary level. Specifically, an original report revealed that SST-KO had elevated GH and glucocorticoid levels as compared with their littermate controls (Low, MJ *et al.* 2001; Zeyda, T *et al.* 2001). Later on, these results were supported by observations from our laboratory showing that the somatotrope and corticotrope axes were altered in a sex-dependent manner in this model (Luque, RM *et al.* 2006b; Luque, RM *et al.* 2007a). Specifically, POMC expression was increased in male SST-KO mice, which was consistent with the in vitro inhibitory effect of SST on POMC expression and ACTH release in both basal and CRF-stimulated states observed in mice (Luque, RM *et al.* 2006b). Thereby, lack of SST at hypothalamic/pituitary levels seems to promote an augmentation in POMC/ACTH tone that evokes an increase in circulating glucocorticoids. Furthermore, increased stomach ghrelin expression, but not hypothalamic and pituitary expression, together with an up-regulation in circulating total ghrelin but not acylated-ghrelin levels

were observed in SST-KO mice as compared with controls (Luque, RM *et al.* 2006b) demonstrating that the regulatory action of SST on ghrelin expression is tissue-specific. In the same report, acylated-ghrelin failed to increase directly ACTH release in mouse pituitary cell cultures (Luque, RM *et al.* 2006b) which, together with other observations suggested that SST-mediated alteration in total circulating ghrelin levels were not directly responsible for the alterations in the hypothalamic-pituitary-adrenal axis (i.e. elevated glucocorticoids levels) observed in SST-KO mice. However, it should be noted that the direct pituitary effect of ghrelin on ACTH release may be dependent of the species and the physiological state of the tissue donor, since it has been reported that ghrelin increases ACTH release in primary pituitary cell culture of baboons (Luque, RM *et al.* 2006b) and in cultures of human corticotropinomas (Martinez-Fuentes, AJ *et al.* 2006). In line with this idea, it has also been suggested that central SST and ghrelin could have a plausible role in the regulation of ACTH release, in that SST inhibits pituitary ghrelin o-acyl transferase (GOAT; the enzyme responsible of the acylation of ghrelin) as well as ghrelin expression in vitro and in vivo (SST-KO) (Gahete, MD *et al.* 2010b), and increased hypothalamic acyl-ghrelin could cause augmentation of NPY with the subsequent inhibition of GABA to finally allow the release of CRF (Cowley, MA *et al.* 2003; Stevanovic, D *et al.* 2007) that will stimulate ACTH release.

As mentioned above, GH levels were found to be elevated in SST-KO (Low, MJ *et al.* 2001; Zeyda, T *et al.* 2001); however, the intrinsic changes produced by lack of endogenous SST on sometotrope-axis were not fully elucidated in these reports. More recently, a study from our group (Luque, RM *et al.* 2007a) reported that both, in vitro and in vivo, pituitaries of SST-KO mice released more basal GH than normal pituitaries, thereby reinforcing the idea of an enhanced capacity of GH release by somatotropes originally proposed by Low et al (Low, MJ *et al.* 2001). Additionally, an increase in hypothalamic GHRH and pituitary GH, GHRH-R and GHS-R expression were also reported in female, but not male, SST-KO mice (Luque, RM *et al.* 2007a) which demonstrated a higher sensitivity of females to SST-absence, which confirmed and extended the sexually dimorphic role of SST on regulation of GH-axis. Interestingly, CST was found to be up-regulated only in male SST-KO suggesting that this peptide may serve a compensatory role in maintaining GH-axis function in SST-KO male mice (Luque, RM *et al.* 2007a). On the other hand, it was found that other systemic signals, such us ghrelin and corticosteroids, were upregulated in male and female SST-KO (Luque, RM *et al.* 2006b; Luque, RM *et al.* 2007a; Zeyda, T *et al.* 2001), and could also

help to increase GH release in mice of both genders. Finally, changes were also observed in the liver, a primary target of GH where it induces synthesis and release of IGF-I through GH-R (Le Roith, D *et al.* 2001). Specifically, Low et al, reported that loss of endogenous SST did not alter expression of IGF-I, however, there were changes in major urine proteins (MUPs) and prolactin receptor (PRL-R) expression (decrease and increase, respectively) that could indicate a "feminization" of SST-KO liver. Moreover, other groups observed that hepatic expression of GH-R and IGF-I, as well as circulating IGF-I levels were elevated only in female SST-KO mice as compared with their controls (Luque, RM *et al.* 2007a), which could be related to enhanced GH signaling in the presence of estrogens (Meinhardt, UJ *et al.* 2007) demonstrating that the sexually dimorphic role of SST on hypothalamic and pituitary GH-axis regulation is extended to other tissues.

Non-human primate model: The olive baboon *(Papio anubis*): a primate model to study pituitary physiology.

To date, most of our understanding of the control of pituitary hormone secretion and its regulation has been generated with the use of laboratory rodents and human (patho)-physiological samples (fetal and tumoral cell cultures). In spite of the wealth of knowledge gathered about regulation of pituitary hormone release with these models, there are still a number of aspects of regulation of pituitary physiology that remain unclear. In this scenario, some non-human primate models could supply normal and experimentally altered pituitaries to ascertain direct actions of regulators of pituitary hormone release. Moreover, these samples might reproduce (patho)-physiological conditions or indicate specific regulation of primate lineage that could not be determined with the use of other models. Accordingly, results obtained with the use of non-human primate models are actually being used for translational research to humans (Braundmeier, AG *et al.* 2009; Comuzzie, AG *et al.* 2003; McClure, HM 1984). However, the choice of a good primate model needs validation of the presence and composition of regulatory systems in the pituitary. In this regard, comparison of sequences of mRNA and predicted proteins of primate with that of humans would indicate the similarity between these two models. Furthermore, it is important to keep in mind that an experimental approach using pituitary cells (primary pituitary cell cultures conditions vs. in vivo conditions) should not alter expression of the regulatory system present in these pituitary cells. A non-human primate model that fulfills all these features is the olive baboon (*Papio anubis*), which it has been used in previous reports to analyze pituitary

physiology (Kineman, RD *et al.* 2007a; Luque, RM *et al.* 2006c). Therefore, the high fidelity at genomic, proteomic and physiological levels shared by this model with humans, together with the in vivo and in vitro conservation of the pituitary regulatory systems, makes the baboon an excellent tool to study the effects of different peptides/hormones on specific pituitary hormone function, which cannot be evaluated in healthy human subjects. Altogether, olive baboons can be considered a suitable, valued non-human primate model to study normal, non-pathological human physiology (Braundmeier, AG *et al.* 2009; Guardado-Mendoza, R *et al.* 2009; Kineman, RD *et al.* 2007a; Luque, RM *et al.* 2006c).

Aims of the study

The two **general aims** of this study were:

1) To study the unique role of SST, CST, their receptors and other related molecular components in the regulation of GH and its hypothalamo-pituitary-metabolic axis, as well as other pituitary-endocrine axes by using mouse models with altered metabolic conditions and mice lacking SST or CST.

2) To establish the precise contribution and mechanisms of action of SST, CST and their receptors, as well as other peptides, such as kisspeptins, and their receptor in the regulation of GH and other pituitary hormone axes in a non-human primate model, the baboon (*Papio anubis*).

These general aims were developed into the following **specific objectives**:

Objective 1: To identify potential new sst5 variants in rodents, related to those identified previously in humans, and perform an initial characterization of these variants in normal mice and in mice models with altered patho-physiological conditions in the GH-axis.

Objective 2: To determine how endogenous SST and its receptors contribute at the hypothalamic, pituitary and stomach levels, to the sex-dependent, metabolic control of GH axis in mice.

Objective 3: To determine whether endogenous CST represents a mere natural analog of SST in the regulation of metabolic/endocrine secretions or whether it plays specific, unique roles, by characterizing CST deficient mice in comparison to SST-KO mice.

Objective 4: To determine the direct actions and the underlying individual sst receptors and intracellular signaling pathways effected by SST and CST to regulate GH secretion from somatotropes in a non-human primate (*P. anubis*) model.

Objective 5: To establish the regulatory effects of kisspeptins on the secretion of LH, GH and other pituitary hormones in primary cultures of pituitary cells from a non-human primate (*P. anubis*) model, and to elucidate the intracellular signaling pathways mediating these effects.

Results and general discussion

In spite of the wealth of knowledge gathered to date on the regulation of GH synthesis and release in many species and under many pathophysiological situations, there still remain a number of actions and players in the systems controlling the somatotropic axis that are uncertain and undiscovered. Indeed, finding new, "atypical" and rare functions for known regulators as well as discovery of novel players and functions should not be unexpected but natural, since there are a number of redundant, complementary and compensatory systems in the organism that ensure the precise control the proper physiology of the endocrine axes and might conceal the unknown and atypical actions carried by those signals and many new factors yet to be discovered. In the aim of unveiling the regulatory actions produced by such specific signals on somatotropic axis, and of helping to better understand some pathological phenomena associated to SST/CST/sst system, we employed several animal models and state-of the art methodologies that generated the results contained in the following studies, which have been structured in six chapters, corresponding to six related scientific manuscripts.

Identification and characterization of new truncated sst5 in rodents (article I)

The existence of sst subtypes additional and distinct to the known ss1-sst5 had been postulated earlier by different groups based on observations that not all actions exerted by SST and/or CST could be explained by the sst subtypes identified at that time (Patel, YC *et al.* 1994b; Tallent, M *et al.* 1996). In this context, our group has recently described the existence of two novel truncated sst5 in humans, named hsst5TMD5 and hsst5TMD4 (Duran-Prado, M *et al.* 2009). Molecular characterization of these novel human receptors was initially carried out using *in vitro* models (cell lines and cell cultures) and a limited set of human samples obtained from pathological tissues. In this study, we sought to identify possible truncated sst5 counterparts in experimental rodents, since these animal models have two important characteristics: 1) Rodents have been extensively used in the last two decades as suitable models to directly investigate the consequences that a given patho-physiologic state can cause on specific tissue and cell function as a result of a metabolic alteration or dysregulation of a gene; and 2) their feasibility to experimental manipulation at physiological level (i.e. fasting, obesity) as well as at the genomic level, by generating genetically modified mice over- or under-expressing the product of interest [i.e. mice lacking SST or CST, mice over-expressing GHRH (MT-hGHRH)]. Accordingly, we used RT-PCR methodology to identify new sst5 truncated variants in the hypothalamus and pituitary of rodents. As a result, we obtained novel variants of rodent sst5 with different size and sequence than full-length sst5, which, like human sst5

variants, are generated by splicing of cryptic introns, and result in new receptors that maintain the same N-terminal region as full-length sst5, but have less transmembrane domains (TMD), and different (and shorter) C-terminal tails. Specifically, we have identified three truncated sst5 variants in mouse with a CDS of 576, 621 and 384 nucleotides and one in rat with a CDS of 381 nucleotides. Mouse sst5 variants would encode proteins of 191, 206 and 127 amino acids, whereas rat sst5 variant would have 115 amino acids. Subsequent topology analysis predicted that mouse sst5 variants possess 4, 2 and 1 TMD, and rat sst5 variant one TMD, and they were therefore named msst5TMD4, msst5TMD2 and msst5TMD1, and rsst5TMD1, respectively. It should be noted that, in striking contrast to their human and rodent counterparts, mouse and rat sst5TMD1 variants showed high interspecific nucleotide (87%) and amino acid sequence identity (86%).

Truncated mouse sst5 receptors also display a distinct intracellular localization compared to msst5, in that the latter is mainly located at plasma membrane as most classical 7TMD receptors, whereas truncated sst5 variants display a predominant cytoplasmic distribution, which is reminiscent to that found for human sst5 truncated variants. These findings suggest that the motifs located in the C-terminal tail would be critical for the intracellular localization and trafficking regulation of sst5 (Hukovic, N *et al.* 1998; Wente, W *et al.* 2005), which, nevertheless, does not necessarily represent an inactivation of the GPCRs, as these processes could be a transient state between ligand-induced endocytosis or heterotypic interaction of receptors (Reubi, JC *et al.* 2000; Stroh, T *et al.* 2006). Indeed, recent evidence supports the notion that GPCRs passage through endosomes is a rather active step, since thyroid-stimulating hormone receptor, parathyroid hormone receptor and sphingosine-1-phosphate receptor 1 are able to actively signal through G-coupled proteins after ligand-induced internalization (Calebiro, D *et al.* 2009; Calebiro, D *et al.* 2010; Ferrandon, S *et al.* 2009; Mullershausen, F *et al.* 2009). Further, lack of the native C-terminal tail of full-length msst5 in the truncated variants does not have to convey a loss of activity, since other GPCR splice variants show distinct properties to their full-length homologues and can associate with intracellular scaffolding/signaling molecules, which allow them to activate other intracellular signaling pathways (Claeysen, S *et al.* 1999; Fagni, L *et al.* 2000; Kilpatrick, GJ *et al.* 1999; Namba, T *et al.* 1993). In line with this, functional studies on CHO-K1 cells transfected with full-length or truncated mouse sst5 variants, revealed that the different receptors exhibited similar, but also distinct and even opposite features in response to SST and CST treatment in terms of $[Ca^{2+}]_i$ kinetics as well as inhibition of forskolin-

induced cAMP levels. Specifically, we first observed that, in keeping with that previously reported by our group and others (Akbar, M *et al.* 1994; Ben-Shlomo, A *et al.* 2005; Duran-Prado, M *et al.* 2009), and despite sst5 being a known, classical inhibitory receptor, both SST and CST increased $[Ca^{2+}]_i$ levels in a msst5-expressing CHO-K1 cells, and did so by generating similar response profiles. Remarkably, in spite of its reduced size, msst5TMD1 essentially mimicked the responses mediated msst5, although to a lower extent. By contrast, whereas msst5TMD4 exhibited a preferential response only to SST, msst5TMD2 increased $[Ca^{2+}]_i$ exclusively in response to CST. These results closely resemble those found for the human truncated sst5 variants, which also displayed selective activation by SST (hsst5TMD4) or CST (sst5TMD5) (Duran-Prado, M *et al.* 2009). This similitude also suggests that specific structural or sequential determinants shared by truncated receptors selectively responsive to SST or CST may help to better understand the unique differential functions of this two highly similar peptides as well as to explore the molecular basis underlying their distinct actions.

On the other hand, truncated mouse sst5 variants were able to inhibit intracellular accumulation of forskolin-induced cAMP levels, an observation that is in contrast to that found for msst5, which was unable to inhibit cAMP levels in response to SST or CST. Specifically, msst5TMD2 and msst5TMD1 inhibited forskolin-induced cAMP levels in CHO-K1 cells exclusively in response to SST, but not to CST. Interestingly, while full-length msst5 and msst5TMD4 could not inhibit forskolin-induced cAMP levels, we observed a non-significant trend for these receptors to enhance cAMP levels, which would be consistent with the stimulatory effect exerted by SST or specific sst5 agonists through mouse and human sst5 on cAMP accumulation in some (Akbar, M *et al.* 1994; Carruthers, AM *et al.* 1999; Cervia, D *et al.* 2003) but not all (Carruthers, AM *et al.* 1999) models.

After identifying the novel mouse sst5 truncated variants and establishing their basic molecular and functional features, the potential physiological relevance of these receptors was explored by use of in vivo murine models representative of diverse patho-physiological states (i.e. fasting, obesity, MT-hGHRH, SST-KO mice). In addition, *in vitro* murine models (i.e. hypothalamic N6 cells and primary pituitary cell cultures) provided information about regulation of the mouse sst5 variants expression. This approach revealed that sst5 variants are down-regulated only in specific pathological states (e.g. catabolic state: fasting) whereas there is no such regulation under opposite conditions (obesity). It also showed that the control of mouse sst5 variants expression is tissue-specific, since their regulation at pituitary level *in vivo* and *in vitro*, in primary pituitary

cell cultures, was closely correlated, whereas a comparable regulation was not observed in hypothalamus *in vivo* or in hypothalamic N6 cells (*in vitro*). Surprisingly, in spite of the fact that SST and CST bind with the same affinity to sst1-5, we observed a ligand-specific regulation of sst5 variants expression, because only CST caused a down-regulation of sst5 and sst5TMD2 in primary pituitary cell cultures. Furthermore, analysis of the role of SST on *in vivo* pituitary sst5 variants expression revealed that SST is a critically required regulator for the fasting-induced fall observed in the msst5 synthesis, whereas truncated sst5 variants expression was down-regulated in the fasted pituitary despite of the lack of SST.

In summary, rodents also express truncated sst5 variants bearing a C-terminal tail that differs from that of full-length msst5. Existence of these new receptors may be of patho-physiological relevance because they are widely distributed at central and peripheral levels, display differential subcellular localization than full-length msst5, and are functional, able to mediate ligand-selective changes in $[Ca^{2+}]_i$ and cAMP production. Furthermore, murine models revealed that expression of sst5 variants is regulated in a tissue-specific manner (central vs. systemic), by changes in hormonal and metabolic environment (i.e. fasting, lack of endogenous SST), and is also regulated in a ligand-dependent manner, since CST but not SST, down-regulated sst5 variants mRNA levels in primary pituitary cell cultures.

SST/CST/sst1-5 expression in SST or CST knock-out mouse (articles II, III)

It is now well established that GH is released in a pulsatile manner that differs markedly between males and females in all mammalian species studied to date (Giustina, A *et al.* 1998; Hartman, ML *et al.* 1993; Jansson, JO *et al.* 1985), and that this sexually-dimorphic pattern of GH exerts gender-specific effects on structural growth, as well as liver function (Waxman, DJ *et al.* 2006). Specifically, GH release in males tends to be highly organized, with high amplitude pulses and low baseline values. In contrast, GH release in females is more disorganized, with increased pulse frequency and elevated baseline values. It is believed that females could have less SST output from its main tissue-sources (hypothalamus and GIT), allowing for an increase in baseline GH release (Giustina, A *et al.* 1998). This notion is based in part on reports showing that female rats and mice have lower SST mRNA levels in the periventricular nucleus and lower immunodetectable SST in the median eminence, compared with males (Chowen, JA *et al.* 2004; Hasegawa, O *et al.* 1992; Low, MJ *et al.* 2001; Murray, HE *et al.* 1999; Nurhidayat *et al.* 1999). Moreover, the importance of SST in maintaining gender-dependent GH pulse

patterns and the importance of the pulse pattern on subsequent GH actions are illustrated by the observation that baseline GH values are increased in male SST-KO mice, and this change is associated with a shift in GH-dependent hepatic gene expression to resemble that of female mice (Low, MJ *et al.* 2001; Meyer, RD *et al.* 2009). GH is also elevated in male and female SST-KO (Low, MJ *et al.* 2001; Luque, RM *et al.* 2007a; Zeyda, T *et al.* 2001), and CST-KO (see below; article III), and female pituitary GH expression is elevated in both models. The apparent role that SST, and CST, plays in the sexually-dimorphic pattern of GH release, coupled with data suggesting that changes in SST/sst expression contribute to the fasting-induced GH release, led us to hypothesize that differential tissue expression of SST/CST/sst-subtypes may contribute to the gender-specific GH pattern observed between genders. Therefore, in order to study the tissue-specific roles of SST/CST/sst in these processes, we evaluated the expression pattern of the SST/CST/sst-subtypes system in whole hypothalamus, pituitary and stomach by quantitative real-time RT-PCR (qrtRT-PCR) in male and female mice, under fed and fasted conditions, in the presence or absence of endogenous SST or CST (wild-type, [+/+] vs. knockout [-/-]).

Our results indicate that, whereas SST was expressed in whole hypothalamus, pituitary and stomach of mice, expression of CST was restricted to hypothalamus. Expression of sst1-5 was detected in all these tissues, although their specific patterns and levels of expression were tissue-dependent. In particular, the more expressed receptor in all tissues was sst2A followed by sst4 ≥ sst1 > sst3 > sst5 ≥ sst2B >> sst5TMD1 = sst5TMD2 in whole hypothalamus, by sst5 ≥ sst3 = sst1 = sst4 > sst5TMD2 >> sst2B >>> sst5TMD1 in pituitary and by sst2B >> sst4 > sst3 > sst1 = sst5 in stomach. It should be noted that no differences were found in the expression levels of whole hypothalamic or stomach SST or sst-subtypes between genders, a finding that lessens the potential role of SST/ssts in these tissues in the sexually dimorphic pattern of GH release. In line with this, other laboratories (Chowen, JA *et al.* 2004; Hasegawa, O *et al.* 1992; Low, MJ *et al.* 2001; Murray, HE *et al.* 1999; Nurhidayat *et al.* 1999), but not all (Bouyer, K *et al.* 2006; Kuwahara, S *et al.* 2004), have observed a higher immunodetectable amount of SST and sst1 in specific hypothalamic regions of male rats as compared with females. Thus, future (neuro)-anatomical studies in these animal models may help to unequivocally ascertain this apparent lack of gender-related differences in SST/ssts at the hypothalamus. On the other hand, we observed differences in SST and sst-subtypes expression between genders in pituitary. Specifically, we detected that sst1 was higher in females, in agreement with that previously reported by some (Señaris, RM *et al.* 1996), but not all authors (Zhang,

WH *et al.* 1999). However, sst2 and sst5 (the most relevant receptors in the control of GH release at pituitary) (Luque, RM *et al.* 2008) were more abundantly expressed in male mice, results that are in line to that previously observed in rats (Kimura, N *et al.* 1998; Zhang, WH *et al.* 1999). In addition, we observed that male mice have elevated sst2B, sst3, sst5TMD2 and sst5TMD1 mRNA levels compared with female mice. Altogether, these results suggest that female pituitary would be less responsive to the actions of SST, an idea that is further supported by our *in vitro* observations showing that SST was more potent in inhibiting GH synthesis and release in primary pituitary cell cultures from male compared to those from female mice.

Since fasting has been shown to enhance GH release in the majority of the mammalian species studied to date [including human and mice (Ho, KY *et al.* 1988; Luque, RM *et al.* 2007b; Rigamonti, AE *et al.* 1998), but with the exception of male rats (Tannenbaum, GS *et al.* 1976)], we sought to determine whether changes in SST/sst-subtypes expression could contribute to the fasting-induced rise in GH release. Our results indicate that fasting-(48h) reduced hypothalamic SST and sst2A, stomach sst2A and sst2B in both, male and female mice, while it caused an overall down-regulation of pituitary sst-subtypes in male (only sst2A was reduced in female). Thus, results from these studies suggest that one component that could contribute to the enhanced GH release observed under fasting conditions, at least in mice, is the generalized reduction in relevant elements of the SST-sst system observed across tissues.

Use of SST-KO (Low, MJ *et al.* 2001; Luque, RM *et al.* 2006b; Luque, RM *et al.* 2007a; Zeyda, T *et al.* 2001), as well as CST-KO (see below; article III), mouse models has enabled to show that SST and CST are required to normally suppress GH release in both males and females, and that these actions may be critical to preserve the gender-dependent pattern of GH release and its subsequent actions. Nevertheless, it is plausible that in SST or CST knock-out mice a compensation by the counterpart neuropeptide occurs that could avoid deleterious effects in physiology induced by the lack of these endogenous peptides. In support to this idea, we have previously shown that hypothalamic CST expression is elevated in male SST-KO mice (Luque, RM *et al.* 2007a), and we have now demonstrated that SST is upregulated in stomach of female of CST-KO (see below; article III). Therefore, the compensatory mechanisms unveiled by knock-out mice, which likely reflect a regulatory interrelationship enabling an counterbalance of these highly homologous neuropeptides, seems to be tissue- and gender-specific, and reveal a higher sensitivity of male hypothalamus to lack of SST and of female stomach to CST deficit.

However, not only SST or CST expression was altered in knock-out mice, but marked changes were also observed in the expression levels of sst subtypes. Thus, sst2A expression was down-regulated in the hypothalamus and pituitary while sst2B mRNA levels were decreased only in pituitary of male SST-KO. In contrast, sst2B expression was upregulated in pituitary of female SST-KO. Additionally, in stomach, sst4 in males and sst1 and sst5 in females were downregulated. Hence, lack of SST has an overall negative effect on sst1-5 expression in SST-KO mice and the most relevant changes were located at hypothalamus and pituitary. To investigate if these changes may be associated with the increased GH release observed in this knockout model, we determined if the direct action of SST can alter sst1-5 expression in these tissues. Results clearly indicated that downregulation in pituitary sst2A/B may be directly mediated by loss of SST in male mice. Finally, we observed that lack of CST did not have any effect in the expression of hypothalamic and stomach sst-subtypes however, it did exerted an overall upregulation in pituitary sst-subtypes expression (sst2A, 3, 5 and sst5TMD1) in female CST-KO mice. Further results using primary pituitary cell cultures indicated that these changes in female CST-KO may be directly mediated by the absence of CST. Thus, when viewed together, these results demonstrate that lack of endogenous SST or CST causes a relevant impact on the regulatory system integrated by SST, CST and sst subtypes, which is tissue- and gender-dependent. Moreover, our data suggest that SST and CST can exert physiologically relevant functions in regulating, gender-dependently the GH axis (and likely other systems), by influencing each other's expression as well as that of their shared sst receptors.

Endocrine/metabolic characterization of cortistatin knock-out mouse (article III)

Inasmuch as CST and SST similarly bind to all sst1-5, and apparently share many actions at different endocrine/metabolic targets (Broglio, F *et al.* 2008; Gahete, MD *et al.* 2008), it has been widely assumed that CST is a simple SST analogue. However, recent studies pointed out that CST exerts specific actions distinct from that of SST. Therefore, we sought to determine whether CST deficiency evoked similar alterations to SST deficiency at pituitary and systemic levels (Low, MJ *et al.* 2001; Luque, RM *et al.* 2006b; Luque, RM *et al.* 2007a; Zeyda, T *et al.* 2001) in the regulation of endocrine hypothalamus-pituitary axes and metabolic endpoints.

Somatotrope cells are the main target of SST actions in pituitary, and deficiency of SST evoked a rise of GH levels (Low, MJ *et al.* 2001; Luque, RM *et al.* 2007a; Zeyda, T *et al.*

2001). Consistent with that previously reported for sst-/- mice, elevated plasma GH levels were found in cort-/- mice, which did not result in significant changes in body weight or linear growth as compared to controls. However, in contrast to that found in female sst-/- mice, IGF-I levels were not altered in cort-/-. Likewise, as in sst-/- (Luque, RM *et al.* 2007a), elevated GH levels in cort-/- were not accompanied by changes in the population of somatotropes, suggesting an increased GH secretory activity of individual somatotropes. However, at variance with that found in SST-KO mice (Luque, RM *et al.* 2007a), the enhanced GH release observed in cort-/- mice *in vivo* was not maintained *in vitro*. This, coupled to our observation that SST but not CST is expressed at the pituitary level in control mice, suggests that circulating CST arising from other tissue sources should account for the changes in GH-output observed *in vivo*.

Results also revealed that female somatotropes are more sensitive to the absence of endogenous CST *in vivo,* since a higher enhancement of GH release was found in female (six-fold) than in male (four-fold) cort-/- mice with respect to their normal littermates. In support to this hypothesis, we found that lack of endogenous CST enhanced expression of pituitary GH only in female cort-/- mice. In addition, although CST inhibited GH release in primary pituitary cell cultures from both male and female mice, it only decreased GH expression in those from female mice (and also female baboon). The ability of CST to inhibit GH release observed herein in mice and baboons is consistent with some (Broglio, F *et al.* 2002a; Deghenghi, R *et al.* 2001a; Grottoli, S *et al.* 2006; Rubinfeld, H *et al.* 2006), but not all (Jeandel, L *et al.* 1998; Luque, RM *et al.* 2006e; Rubinfeld, H *et al.* 2006), reports showing that CST inhibits *in vitro* GH release. Analysis of the expression of several hypothalamic peptides known to influence somatotrope function revealed that changes in GH synthesis and release found in cort-/- mice were independent of changes in hypothalamic expression. In contrast, pituitary somatostatin receptor mRNA levels were up-regulated, albeit only in female cort-/- mice, which could be interpreted as a compensatory mechanism to decrease the particularly elevated GH synthesis and release observed in these animals. Interestingly, examination of systemic/peripheral signals revealed elevated glucocorticoid levels and down-regulated insulin levels in cort-/- mice, changes that would favor elevated GH release observed in male and female mice (Luque, RM *et al.* 2011; Luque, RM *et al.* 2009 ; Park, S *et al.* 2004). Of note, an up-regulation of ghrelin and GOAT mRNA levels in stomach, as well as elevated circulating levels of total and acylated-ghrelin were observed only in females, which would likely contribute to the enhanced sensitivity of the female GH-axis to CST loss (i.e. significant up-regulation of GH synthesis and release as compared with male)

(Garcia, A *et al.* 2001). In all, our results demonstrate that changes observed in the GH-axis of cort-/- mice are, at least in part, substantially different to those observed in sst-/- mice (Low, MJ *et al.* 2001; Luque, RM *et al.* 2007a), thus suggesting that CST is not a mere natural analogue of SST in regulating gender-dependent GH-axis and metabolic/endocrine GH-related secretions.

As mentioned above, insulin levels in male and female cort-/- mice were found to be decreased compared with their cort+/+ controls, although this was not associated with an improvement in the response to insulin tolerance tests (ITT). In fact, the responses to glucose and insulin (GTT and ITT) were deteriorated in male cort-/- mice compared with their cort+/+ littermates, unveiling an overall impairment in insulin-mediated glucose clearance, while GTT and ITT responses in female cort-/- mice, as in male and female SST-KO, were similar to their respective controls. We hypothesized that the elevated circulating levels of ghrelin found in male and female sst-/- (Luque, RM *et al.* 2006b) as well as in female cort-/- mice could impart a protective role in pancreatic function, because the non-acylated form of ghrelin has been previously shown to exert a favorable influence on insulin sensitivity and glucose homeostasis (Gauna, C *et al.* 2004; Granata, R *et al.* 2010; Sangiao-Alvarellos, S *et al.* 2010). Taken together, our results disclose a previously unknown [albeit suspected (Gottero, C *et al.* 2004)] involvement of CST in the control of insulin/glucose homeostasis, which may be physiologically relevant, differs from the related actions of SST, and shows a gender divergence that may possibly entail a distinct participation of the ghrelin system in male and female mice.

We next studied whether endogenous CST plays a role in control on the lactotrope axis. Surprisingly, we found that circulating PRL levels were markedly down-regulated in both, male and female cort-/- mice as compared to their cort+/+ controls. This decrease in plasma PRL levels was not associated to any changes in the proportion of lactotrope cells or PRL expression in pituitary; however, we found that secretory capacity of individual lactotropes was reduced, since primary pituitary cell cultures of cort-/- released less PRL under basal conditions.

In striking support of a direct stimulatory role of CST in regulating PRL release were our results showing that CST potently increased PRL release in primary pituitary cell cultures from both mice and baboons. These data are in contrast to some of the limited data available in this area, indicating that of CST inhibits PRL release from cultured prolactinomas (Rubinfeld, H *et al.* 2006) similar to SST, which inhibits PRL release from fetal human, rat and fish cultured pituitary cells (Enjalbert, A *et al.* 1982; Grau, EG *et al.* 1987; Shimon, I *et al.* 1997a). However, our findings are strongly supported by results

from a recent study showing that CST increases PRL release *in vivo* in male rats (Baranowska, B *et al.* 2009). Moreover, indirect evidence indicating that CST could be increasing PRL release in humans has also been reported, in that ghrelin-induced PRL increase in normal human subjects was significantly enhanced when CST was administered simultaneously (Broglio, F *et al.* 2002a). It should be emphasized that the positive actions of CST on lactotrope function are in striking contrast with the inhibitory effect exerted by SST on PRL secretion. Accordingly, it was reasonable to think that the unique stimulatory effect of CST on PRL release should be exerted through a different receptor, not shared by SST. Interestingly, it has been reported that in human pituitary CST, but not SST, binds with high affinity to GHS-R1a, the receptor for ghrelin (Broglio, F *et al.* 2007; Deghenghi, R *et al.* 2001b), and this peptide, in turn, is known to stimulate PRL secretion (Broglio, F *et al.* 2002a; Petersenn, S 2002; Rubinfeld, H *et al.* 2004). Indeed, when CST was co-administered with an antagonist of GHS-R1a, BIM-28163, CST-induced PRL release from baboon pituitary cell cultures was completely blocked, thereby indicating that CST stimulates PRL release via GHSR1a.

Analysis of the corticotropic axis in cort-/- mice revealed that CST also influences its regulation in a relevant manner. Thus, female cort-/- mice showed higher circulating ACTH levels and POMC expression, whereas an increase in plasma glucocorticoid levels was observed in both, male and female cort-/- as well as in SST-KO (Luque, RM *et al.* 2006b; Zeyda, T *et al.* 2001). The increase in ACTH release and POMC expression in female cort-/- mice was not correlated with a higher population of corticotropes as compared with their controls, however, it was associated with an enhanced ACTH-secretory vesicle rate in primary pituitary cell cultures of female, but not male, cort-/- mice under basal conditions. In addition, the results observed *in vivo* were supported by our *in vitro* data showing that CST treatment decreased ACTH release from pituitary cell cultures of male and female mice and baboons, whereas it only inhibited POMC expression in female mice (and baboons). The gender-dependent differences observed in the corticotrope axis of cort-/- mice could not account for by changes in hypothalamic expression of primary regulators of ACTH secretion. However, an upregulation of the ghrelin system observed in female cort-/- mice (acylated and total ghrelin levels and stomach ghrelin and GOAT expression) may contribute to the selective increase in circulating ACTH observed in female cort-/-, since it has been shown that acylated ghrelin can positively regulate corticotrope function (Bakker, RA *et al.* 2006; Broglio, F *et al.* 2002a; Gottero, C *et al.* 2004; Luque, RM *et al.* 2006b; Martinez-Fuentes, AJ *et al.* 2006; Stevanovic, D *et al.* 2007; van der Lely, AJ *et al.* 2004). Finally, analysis of the

gonadotrope and thyrotrope axes reveales that CST deficiency did not alter the normal function of these cell types.

Thus, when viewed as a whole, our results unveil that CST exerts unique, unpredicted regulatory actions on the pituitary-metabolic axis, which are distinct to those previously established for SST. In particular, the unique, unexpected actions of CST on the control of PRL, ACTH, and GH-related glucose/insulin homeostasis, and the fact that these actions, not mimicked by SST, are strongly gender-dependent offer new grounds to investigate the hitherto underestimated physiological relevance of CST in the regulation of endocrine and metabolic process.

Direct effect of SST, CST and sst-analogs on GH secretion of a non-human primate (article IV)

It is well established that both SST and CST act at the pituitary to inhibit GHRH-stimulated GH synthesis and release, as well as basal GH secretion, depending on the species studied and the dose tested (Bakker, RA *et al.* 2006; Broglio, F *et al.* 2002a; Broglio, F *et al.* 2008; Castaño, JP *et al.* 2005; Gahete, MD *et al.* 2008; Giustina, A *et al.* 1998; Muller, EE *et al.* 1999). However, our group has demonstrated that low doses of SST (and/or CST) can function as a true GH-releasing factor in porcine pituitary cell cultures (Castaño, JP *et al.* 2005; Castaño, JP *et al.* 1996; Luque, RM *et al.* 2006a; Luque, RM *et al.* 2006e; Ramirez, JL *et al.* 1998; Ramirez, JL *et al.* 1997). In humans, our understanding of the direct pituitary effect of SST on GH secretion has been based on studies conducted in primary cell cultures established from human fetal pituitaries or GH-producing pituitary adenomas which have provided complex, and at times contradictory results (Florio, T *et al.* 2003; Gahete, MD *et al.* 2008; Giustina, A *et al.* 1998; Goodyer, CG *et al.* 1993; Goodyer, CG *et al.* 1987; Ishibashi, M *et al.* 1984; Ren, SG *et al.* 2003; Rubinfeld, H *et al.* 2006; Shimon, I *et al.* 1997a; Shimon, I *et al.* 1997b). In fact, some studies suggest that the stimulatory actions of SST may not be limited to the pig somatropes, as it has been reported, but not emphasized in the literature, that SST and/or SST-analogs can induce a paradoxical GH release in certain GH-secreting adenomas *in vitro* (Daniels, M *et al.* 1991; Florio, T *et al.* 2003; Matrone, C *et al.* 2004; Murray, RD *et al.* 2004; Shimon, I *et al.* 1997a).

The positive action of SST on GH release may also be related to so-called "post-SST rebound" response. Indeed, it is well-known that circulating GH levels *in vivo* rise following discontinuation of SST infusion in humans and animal models (Cella, SG *et al.* 1996; Clark, RG *et al.* 1988; Dickerman, Z *et al.* 1993; Giustina, A *et al.* 1998; Hindmarsh,

PC *et al.* 1991; Jaffe, CA *et al.* 1996; Robinson, IC *et al.* 1990; Tannenbaum, GS *et al.* 1989). This "rebound" release has been classically attributed to SST stimulation of GHRH neuronal activity, where upon sufficient degradation of circulating SST concentrations, GHRH is free to induce GH release. However, as mentioned above, low doses of SST and CST [i.e., within the range of those measured in porcine portal plasma during trough periods; (Drisko, JE *et al.* 1998)], can acutely stimulate GH release from primary pig pituitary cell cultures (Castaño, JP *et al.* 2005; Castaño, JP *et al.* 1996; Luque, RM *et al.* 2006a; Luque, RM *et al.* 2006e; Ramirez, JL *et al.* 1998; Ramirez, JL *et al.* 1997) suggesting that, a component of the GH rebound effect observed *in vivo* may be due to degradation of circulating SST to concentrations within the stimulatory range.

Although "post-SST rebound" responses have been described also in men and children (Dickerman, Z *et al.* 1993; Hindmarsh, PC *et al.* 1991; Jaffe, CA *et al.* 1996), the potential relevance of these observations to the human (or, in general, primates) are as yet unknown. In fact, there are no studies available using primary pituitary cell cultures from normal adult humans or from non-human primate (a model with many similarities at the physiological and genomic levels to humans) describing the actions of low-dose SST or CST (Comuzzie, AG *et al.* 2003; Kineman, RD *et al.* 2007a; McClure, HM 1984). Accordingly, the present work was designed aimed at determining if low doses of SST (and CST) can directly stimulate GH release in a primate model (*Papio anubis;* baboon) and, if so, to delineate the intracellular signaling pathways involved, and to ascertain, using synthetic selective sst-subtype agonists and antagonists, the precise contribution of individual sst subtypes in mediating the inhibitory and stimulatory actions of SST on GH release.

Our results clearly showed that high (micromolar), but not low (picomolar) doses of SST inhibited GHRH and ghrelin stimulated GH release. In fact, whereas high doses of SST did not alter basal GH secretion, low doses of SST (and CST) dramatically increased GH secretion. Surprisingly, an additive effect on GH secretion was observed when low-dose SST (10^{-15}M) was simultaneously applied with ghrelin, but not with GHRH (10^{-8}M). Analysis of signaling mechanisms employed by low-dose SST alone or combined with ghrelin to induce GH release revealed that these effects were probably mediated by an increase in the production of intracellular cAMP, a finding that is reminiscent to that previously observed for porcine primary pituitary culture cells (Ramirez, JL *et al.* 2002). Indeed, our results were further supported by the use of inhibitors of intracellular signaling pathways, which revealed that blockade of the adenylate cyclase/cyclic AMP/protein kinase A (AC/cAMP/PKA) pathway, as well as nitric oxide synthase (NOS)

and intracellular calcium influx completely abolished the stimulatory effect of low dose SST on GH release. These results demonstrate that the stimulatory action of SST on GH release involves a particular, intricate set of signaling cascades that are partially shared but also distinct to those employed by typical GH-releasing factors like GHRH and ghrelin, and should be activated by activation of specific receptor mechanisms (see below).

The physiologic relevance for the biphasic effects of SST (i.e., GH suppression at moderate to high doses and GH stimulation at low doses) remains to be determined. However, based on the concentration patterns of SST released into the pituitary circulation, as determined by hypophyseal portal blood sampling in rats, pigs and sheep (Drisko, JE *et al.* 1999; Fletcher, TP *et al.* 1996; Plotsky, PM *et al.* 1985), it seems plausible that SST input to the pituitary can fall to levels observed to be stimulatory to GH release *in vitro*. Therefore, our results showing that SST, at low concentrations, can directly stimulate GH release in primary pituitary cell cultures of baboons suggest that it is conceivable that direct stimulatory actions of SST may contribute to the rebound GH release, observed in human, following SST infusion withdrawal.

Our next aim was to determine the precise contribution of individual sst subtypes to the stimulatory and inhibitory effects caused by SST on GH release. Use of specific agonists and/or antagonists for the receptor subtypes sst1-sst5 revealed that only sst1 and sst2 were able to inhibit GH release stimulated by GHRH or ghrelin. Interestingly, sst2 agonists were also able to inhibit basal GH release, which contrasted to the inability of a high dose of SST to alter basal GH release. This difference could be related to receptor-receptor interactions. Indeed, whereas sst2 agonists do not induce sst2 internalization if sst5 is coexpressed in the same cell (Kumar, U *et al.* 1997; Mezey, E *et al.* 1998), SST treatment did cause internalization of sst2 (Sharif, N *et al.* 2007) thus rendering this receptor unavailable to induce a more profound or lasting inhibition of GH release. Furthermore, we observed that a high dose of sst5 agonists did not inhibit stimulated GH release; however, at low doses, sst5 agonists closely reproduced the stimulatory effect evoked by low dose of SST on GH release. These results compare well to those previously reported by our group in pigs (Luque, RM *et al.* 2006a) suggesting that the receptors involved in the dual, inhibitory (sst1 and sst2) and stimulatory (sst5), effect of SST are conserved across species. Of note, we demonstrated for the first time that the presence of specific sst5 antagonists completely block the stimulatory effects evoked by low-dose SST or sst5 agonists on GH secretion, thus providing unequivocal support to the idea

that sst5 is the receptor involved in this paradoxical stimulatory effect of low doses of SST.

An addition set of studies were devised at exploring the specific signaling mechanisms employed by sst2 and sst5 agonists to act on somatotropes, and to determine if sst agonists induced their effects by causing similar receptor-mediated signaling than SST (Millar, RP *et al.* 2010). Specifically, use of inhibitors for AC/cAMP/PKA signaling pathway completely blocked the inhibitory effect of sst2-agonist and also abolished the stimulatory effect of sst5-agonist on basal GH release. The involvement of this signaling pathway in mediating sst2 and sst5 agonist effects on GH release was supported by the fact that sst2-agonist inhibited while sst5-agonist increased intracellular cAMP levels in baboon primary pituitary cell cultures. The results for sst2 are in close agreement with the commonly accepted notion that sst couple to Gαi to cause their effects (Patel, YC *et al.* 1994a). On the other hand, coupling of sst5 to Gαi is still controversial, because it has also been shown that sst5 might be coupled to Gαs (Akbar, M *et al.* 1994; Carruthers, AM *et al.* 1999; Cervia, D *et al.* 2003; Ramirez, JL *et al.* 2002). In fact, our results are supported by previous reports indicating that CHO-K1 cells transfected with human sst5 respond to SST treatment with an increase in intracellular cAMP accumulation, where this action could be blocked by treating the cells with a dominant negative negative Gαs (Gαs acetyl 354–372) (Carruthers, AM *et al.* 1999). When these and previous results are taken together, it is clear that, although much work remains to be done to fully understand the relevance and underlying mechanisms of the dose-dependent, biphasic effects of SST on GH release, our current data provides solid evidence that this phenomenon is indeed real and can occur in a species relevant to humans.

Regulation of sst-subtypes, GHRH-R and GHS-R by high and low doses of SST, GHRH and ghrelin in primate pituitary cell cultures (Article V)

The response of somatotropes and other pituitary cell types to their regulatory factors is strongly dependent on the type and amount of receptors that are available at the cell membrane at a given point in time. Accordingly, regulation of receptor expression represents a relevant mechanism to control cell function. In this context, it has long been known that endogenous ligands can induce changes in the expression of their own receptors (homologous regulation) as well as in that of other related receptors (heterologous regulation) in order to reduce or increase their signaling and thereby

modulate their subsequent effects (Luque, RM *et al.* 2004a; Luque, RM *et al.* 2004b). For instance, it has been previously reported that SST can induce an overall upregulation of sst-subtypes expression in pituitary cells (Berelowitz, M *et al.* 1995; Bruno, JF *et al.* 1994a; Luque, RM *et al.* 2004b; Park, S *et al.* 2003; Presky, DH *et al.* 1988), and also, that GHRH and ghrelin can decrease the expression of their receptors (Kineman, RD *et al.* 2007a; Luque, RM *et al.* 2004a). However, these effects seem to be highly dependent on the species considered as well as on a number of (patho)physiological elements, including duration of stimulus, etc. (Bruno, JF *et al.* 1994a; Bruno, JF *et al.* 1994b; Kineman, RD *et al.* 2007a; Luque, RM *et al.* 2004a; Luque, RM *et al.* 2004b; Park, S *et al.* 2003). Since the study of this regulation in normal human pituitary does not appear as an attainable goal, in the present work we took advantage of a non-human primate model, the baboon (*Papio anubis*), closer to human physiology and genomics than the commonly employed laboratory rodents, to explore the *in vitro* response of pituitary GHRH/ghrelin/SST receptor expression to their ligands. In this regard, it was of particular interest to analyze the effects of two distinct doses of SST that have been shown to exert an opposite, stimulatory/inhibitory action in GH release from pig (Castaño, JP *et al.* 2005; Castaño, JP *et al.* 1996; Luque, RM *et al.* 2006a; Ramirez, JL *et al.* 1998; Ramirez, JL *et al.* 1997) and baboon somatotropes (see article IV). Results obtained herein comprise the first evidence on the regulation of receptors directly involved in GH release by somatotropes (sst-subtypes 1, 2 and 5, GHRH-R and GHS-R) in a primate model. Specifically, we observed that high doses of SST evoked an overall increase in sst-subtypes expression which is consistent with previous results in other species, including pigs and rats (Berelowitz, M *et al.* 1995; Bruno, JF *et al.* 1994a; Luque, RM *et al.* 2004b; Presky, DH *et al.* 1988). On the other hand, low dose SST (which stimulates GH release), evoked an increase of sst1 and a down-regulation of sst5 expression, which is reminiscent to that observed in porcine pituitary cells (Luque, RM *et al.* 2004b). Therefore, modulation of sst-subtypes in response to different concentrations of its natural ligand seems to be conserved across species, suggesting that this event might represent a physiologically relevant response of the somatotropes to SST. Interestingly, although high-dose SST did not alter pituitary GHRH-R or GHS-R expression, at a low dose SST inhibited GHRH-R mRNA levels.

Analysis of the regulation of GHRH-R and GHS-R in response to GHRH and ghrelin revealed that these receptors were down-regulated by their respective endogenous ligand. Moreover, while both peptides similarly increased sst1 and did not alter sst2 expression, only GHRH was able to inhibit sst5 expression. Because we have previously

reported that the effects of GHRH and ghrelin on baboon GH release are mainly mediated through an activation of AC/cAMP/PKA and PLC/IP/PKC, respectively, we sought to determine whether the regulation of the expression of pituitary receptors evoked by these ligands were mediated via these intracellular signaling routes. As suggested, effects of GHRH in the regulation of all pituitary receptors studied were mimicked by forskolin (an activator of AC) while the effects of ghrelin were reproduced by TPA (phorbol esters; an activator of PKC), thereby suggesting that all these changes were mediated via AC/cAMP and PLC/PKC, respectively. Of note, our results reveal that an increase in cAMP levels exerted by either GHRH or low-dose SST would be responsible not only for the elevation of GH release observed in response to these peptides, but also for the precise modulation of the expression of the sst-subtypes, GHRH-R and GHS-R observed in primate pituitary cell cultures.

In a final set of studies, we explored the individual effects of sst1, sst2 and sst5 signaling on the regulation of their expressions by using selective agonist for each of these receptors (Rohrer, SP *et al.* 1998; Rohrer, SP *et al.* 2000). Results obtained indicated that sst1 agonist did not modify sst1, sst2 or 5 mRNA levels. Converesely, sst2 agonist (which reduced basal GH release) did induce a clear down-regulation of its own receptor, which was in striking contrast with previous results observed with SST. These results suggest that the specific signaling pathways or molecular events activated by this sst2-agonist might be different to that activated by SST, an idea that is supported by a previous report (Sharif, N *et al.* 2007) showing that sst2 is retained (not internalized) at the plasma membrane in response to sst2 agonist challenge in cells that co-express sst2 and sst5 [as occurs in somatotropes; (Kumar, U *et al.* 1997; Mezey, E *et al.* 1998)].

Interestingly, treatment with high or low dose of sst5-agonist (which, respectively, did not alter and increase basal GH release) induced opposite effects on sst2 and sst5 expression. In particular, high doses of sst5 agonist reduced sst2 and increased sst5 expression, while low doses of sst5 agonist increased sst2 and decreased sst5 mRNA levels. These observations may not be atypical and could be of physiological relevance for somatotropes, in that activation of sst5 by low doses of its ligand potently increase GH release, and therefore, somatotropes may respond to this challenge by increasing the expression of the main receptor involved in inhibition of GH release (sst2) as well as by decreasing that of the stimulatory receptor (sst5). On the other hand, activation of sst5 by high doses of its ligand could activate other signals and/or other sst-subtypes (inhibitory sst1 or sst2), which may be interpreted by somatotropes as an overall inhibitory input that would elicit an opposite response: increase in sst5 expression and

decrease of sst2 mRNA levels. In all, our results suggest that this opposite regulation of sst2 and sst5 in response to different doses of sst5 agonist could represent an inner counterbalance of somatotrope cells to facilitate or avoid excessive GH release. These results may be (patho)-physiologically relevant in humans, since the amount and ratio between pituitary sst2 and sst5 expression in human GH-secreting adenomas could be a key factor for the hormonal control of these patients in response to octreotide (a preferential agonist for sst2 and sst5) treatment (Taboada, GF *et al.* 2007; Taboada, GF *et al.* 2008).

Hence, in addition to the well-defined role of SST, GHRH and ghrelin in modulating GH release, these primary regulators of somatotrope function act through distinct signaling pathways to exert both homologous and heterologous regulation of receptor expression, a mechanism that may contribute significantly to modulate the somatotrope response to acute ligand stimulation and thus, would provide an additional layer of complexity to the regulatory mechanisms required to maintain circulating GH levels in a normal range.

Direct effects of Kisspeptin on pituitary hormone secretions of a non-human primate (Article VI)

Kisspeptins (kp), a family of peptides encoded by the Kiss1 gene, and their receptor Kiss1r were first identified by their anti-metastatic actions (Kotani, M *et al.* 2001; Ohtaki, T *et al.* 2001), but have emerged as a key regulatory system for the reproductive axis, where it mainly acts by controlling hypothalamic GnRH release. Recently, kp has been proposed to exert additional regulatory functions at the pituitary and this idea is supported by several observations: 1) Kp is present in ovine and monkey hypophysial portal blood, suggesting that the pituitary gland may be a target of kp (Ramaswamy, S *et al.* 2009; Smith, JT *et al.* 2008), 2) Kp/Kiss1r are highly expressed in pituitary of several species, and is differentially regulated by the steroid milieu, and 3) Kp can stimulate LH secretion in primary pituitary cultures from several species (mouse, rat, pig, cow, goldfish) (Richard, N *et al.* 2009; Roa, J *et al.* 2008). Moreover, kp has unexpectedly been found to directly stimulate GH release from rat, cow, and goldfish somatotropes (Ezzat, AA *et al.* 2010; Gutierrez-Pascual, E *et al.* 2007; Kadokawa, H *et al.* 2008a; Kadokawa, H *et al.* 2008b; Richard, N *et al.* 2009). Most recently, our laboratory has found that kp/Kiss1r is present in the normal human pituitary, and that it can be lost in pituitary adenomas, where kp can exert functional roles (Martinez-Fuentes, A *et al.* 2011). Taking together all these data, it seems increasingly plausible that kp might act as

endocrine/autocrine/paracrine signal in modulating hormonal secretions directly at the pituitary. Unfortunately, it is not clear whether the actions of kp observed in animal models would extend to the normal human pituitary. In an attempt to further clarify the role Kiss1/Kiss1r plays in directly regulating the secretion of pituitary hormones, the current study tested the effects of kp-10 on synthesis and release of all pituitary hormones in primary pituitary cell cultures from baboons (*Papio anubis*), a model very close to humans at the physiologic and genomic levels.

Our results revealed that kp-10 increased LH and GH release in a dose- and time-dependent fashion but did not affect FSH, ACTH, PRL or TSH secretion. The actions of kp were not only restricted to hormone release since kp-10 challenge was also able to up-regulate the expression of pituitary LH (at 12 and 24h) and GH (at 24h). In addition, we also observed that kp-10 enhanced hormonal response to the major regulators of LH (GnRH) and GH (GHRH) release, suggesting that kp-10 activates distinct intracellular signaling pathways than GnRH and GHRH, at least in part, to release LH and GH, respectively. In fact, use of inhibitors for specific key intracellular signaling routes revealed that kp-10 signals through phospolipase C/inositol phosphate/protein kinase C (PLC/IP/PKC), mitogen-activated protein kinases (MAPK), intracellular calcium mobilization, mammary target of rapamycin (mTOR) and phosphatidylinositol-3 kinase (PI3K) to trigger LH release, a complex set of signaling routes which, remarkably parallels that found previously to mediate the actions of kp in GnRH neurons (Messager, S *et al.* 2005; Navarro, VM *et al.* 2005; Roa, J *et al.* 2009). However, these signaling routes activated by kp-10 in the pituitary are partially distinct to that activated by GnRH at the pituitary level to increase LH release [(Stojilkovic, SS *et al.* 1994) and preset study] which might explain the enhanced response of LH release by the co-treatment kp-10 and GnRH. It is interesting to note that whereas the stimulatory effects of kp-10 and GnRH alone on LH release were comparable, we noticed that the effect of kp-10 alone on GH release was clearly weaker than that exerted by GHRH or ghrelin alone. Yet, as mentioned above, kp-10 was able to significantly enhance the stimulatory effect of GHRH, but not ghrelin, suggesting that kp-10 and ghrelin share a set of common intracellular signaling pathways, i.e. PLC/IP/PKC, MAPK, and extracellular calcium influx through L-type channels (Kineman, RD *et al.* 2007a), which differ from those activated by GHRH, (i.e. AC/cAMP/PKA and extracellular and intracellular calcium) (Kineman, RD *et al.* 2007a).

One additional, important issue examined in our study relates to the relationshiup between sex steroids and the kp/Kiss1r system. Sex steroids are well-known key factors

for maintaining physiologic patterns of gonadotrope and somatotrope function (Shibasaki, HI *et al.* 1986; Tang, LK *et al.* 1982). Accordingly, we analyzed the possible effects of estrogen environment (estradiol; E2) on kp-10 stimulated pituitary hormone release. Our results demonstrated that E2 enhanced the relative response of LH to Kp-10, alone or in combination with GnRH and it also served to enhance the relative GH-releasing effect of Kp-10 alone and in combination with GHRH, as compared to E2-free control cultures, suggesting that sex steroids sensitize gonadotrope and somatotrope responsiveness to the direct action of Kp. Altogether, our results indicate that the primate pituitary contains a functional kp/Kiss1r signaling system, which is dynamically regulated and is influenced by estrogens, and that could relevantly contribute to the normal maintenance of the physiological control of gonadotropic/reproductive and somatotropic/growth axes.

In sum, the body of information generated in the present studies supports the general notion that the SST/CST/sst system represents a growing family of ligands and receptors, which in spite of their extraordinary similitudes (at the level of both, ligands and receptors), can exert highly specific actions through versatile ligand-receptor interactions. This new information could help to delineate the differential functions of these similar peptides and their mechanisms of action, and shed new light on the potential usefulness of the knowledge gained, whose exact physiological relevance is now starting to be unveiled and appreciated by use of new animal models and experimental approaches. Indeed, by combining novel experimental procedures and original results in mice and primates, we generated direct evidence for specific actions of SST, CST and associated peptides as well as for crosstalk among the SST/CST and ghrelin systems on pituitary physiology with potential interest, hopefully, for human physiology and translational medicine.

General Conclusions

1.- Rodent posses truncated variants of the sst5 receptor (sst5TMD4, sst5TMD2 and sst5TMD1 in mice and sst5TMD1 in rat), whose expression is distinctly distributed specifically regulated across tissues in a hormonal, metabolic and ligand (SST/CST)-dependent manner. They also display differential subcellular distribution that full-length sst5, and mediate ligand-selective (SST vs. CST) intracellular signaling. These newly identified rodent sst5 variants represent valuable experimental tools and may be of (patho)physiological relevance.

2.- SST and its receptors influence the marked sexually-dimorphic pattern of GH release and the fasting-induced rise in GH by undergoing specific changes in their tissue- and sex-dependent expression, which involve direct responses to SST, IGF-I and glucocorticoids.

3.- Cortistatin is not a simple natural analog of somatostatin in regulating gender-dependent, metabolic/endocrine secretions. Indeed, CST exerts specific gender-dependent actions on GH and ACTH axes not shared by SST, stimulates prolactin release through GHS-R1a signaling, and its deficiency, but not that of SST, alters glucose homeostasis. Hence, CST could play specific, physiologically relevant functions in regulating endocrine-metabolic homeostasis.

4.- Both SST and CST stimulate GH release at low, subnanomolar concentrations by specifically activating adenylyl cyclase signaling via sst5, thus providing a molecular basis for low doses of these peptides contrinuting to the well-known rebound GH release observed in humans following SST infusion withdrawal.

5.- GHRH, ghrelin and SST act through distinct mechanisms to exert a homologous and heterologous regulation of their receptors' expressions, likely contributing thereby to modulate the normal responses of somatotropes to their main regulatory factors.

6.- Kisspeptins can regulate directly primate pituitary function by acting on somatotropes and gonadotropes to stimulate GH and LH expression and release, via specific, cell type- dependent cascades of intracellular signaling.

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Article I

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Identification and characterization of new functional truncated variants of somatostatin receptor subtype 5 in rodents

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Abstract Somatostatin and cortistatin exert multiple biological actions through five receptors (sst1-5); however, not all their effects can be explained by activation of sst1-5. Indeed, we recently identified novel truncated but functional human sst5-variants, present in normal and tumoral tissues. In this study, we identified and characterized three novel truncated sst5 variants in mice and one in rats displaying different numbers of transmembrane-domains [TMD; sst5TMD4, sst5TMD2, sst5TMD1 (mouse-variants) and sst5TMD1 (rat-variant)]. These sst5 variants: (1) are functional to mediate ligand-selective-induced variations in $[Ca^{2+}]_i$ and cAMP despite being truncated; (2) display preferential intracellular distribution; (3) mostly share full-length sst5 tissue distribution, but exhibit unique differences; (4) are differentially regulated by changes in hormonal/metabolic environment in a tissue- (e.g., central vs. systemic) and ligand-dependent manner. Altogether, our results demonstrate the existence of new truncated sst5-variants with unique ligand-selective signaling properties, which could contribute to further understanding the complex, distinct pathophysiological roles of somatostatin and cortistatin.

Keywords Somatostatin receptor · Truncated receptor · Cortistatin · Mouse models · Metabolism

Abbreviations

SST	Somatostatin
CST	Cortistatin
sst	Somatostatin receptor
TMD	Transmembrane domain
HPT	Hypothalamus
PIT	Pituitary
DIO	Diet-induced obesity
MT-hGHRH	Metallothionein promoter-human growth hormone releasing hormone
SST-KO	Somatostatin knock-out
3xHA	3x hemagglutinin
qrtRT-PCR	Quantitative real-time retrotranscriptase-polymerase chain reaction

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Introduction

Somatostatin (SST) is a well-known pleiotropic polypeptide, which is widely distributed throughout the organism and exerts a number of (patho)-physiological functions, including inhibition of endocrine and exocrine secretions and of tumoral cell growth, modulation of neurotransmission, regulation of metabolic, digestive and immune functions. To exert its multiple functions, SST can act through a family of five G-protein couple receptors (GPCR) with seven transmembrane domains (TMD) termed sst1–5 [1–5]. The ssts also seem to mediate most actions of cortistatin (CST), a peptide with strong homology to SST that shares the majority, but not all of the biological actions of SST [1, 6–10]. In spite of the wealth of knowledge gathered in recent years on the patho-physiology of SST/CST and their receptors, there are still a number of unsolved questions. This has led several authors to propose the existence of additional SST or CST receptors and/or signaling mechanisms, either related or not to the sst family, to explain their findings, which indicated that SST and CST can cause distinct effects at both central and peripheral levels [7, 10–14]. However, the existence of such postulated additional SST/CST receptors, or that of specific receptors for SST or CST has not been clearly demonstrated so far, although there is some evidence suggesting that MAS related gene receptor (MgrX2) as well as the GH secretagogue receptor (GHS-R1a) may play such a role by selectively binding CST, and not SST [13, 15–17].

After their discovery, initial studies on seven TMD domain GPCRs were mainly focused on the region of the receptor with the most obvious implications in ligand binding (i.e., the N-terminus), whereas the C-terminal region received somewhat less attention. However, the GPCR C-terminus is now recognized as a critical domain for the regulation of GPCR functions, and over the last years, it has been widely demonstrated that many splice variants of a given GPCR show sequence variations within the C-terminal domain, as is the case for the receptors for growth hormone-releasing hormone (GHRH-R) and gonadotropin-releasing hormone (GnRH-R) [18–22]. In fact, such a diversity of functions of the GPCR C-terminus and its related splicing processing mechanism has led to term this region the ‘magic tail’ [18]. Furthermore, there is increasing evidence that the splice mechanism of GPCR at the C-tail can also result in truncated receptors with fewer than the typical seven TMDs, as has been shown for the receptors for prostaglandin (EP1-4), corticotrophin-releasing hormone, GHRH and gonadotropin-releasing hormone [20–25]. In this context, our group has demonstrated recently the existence of two new truncated but functional splice variants of the human sst5, of five and four TMDs, accordingly termed sst5TMD5 and sst5TMD4. These receptors show a unique expression pattern in normal tissues as well as in different pituitary tumor types and display distinct responses to SST and CST [26].

Laboratory rodents are widely used as animal models to study the consequences that a patho-physiologic state (i.e., fasting, obesity) can cause on specific tissue and cell functions because of their easy experimental manipulation. Likewise, rodents, especially mice, are ideally suited to study the pathophysiological importance of gene products because of the feasibility to generate genetically modified mice over- or under-expressing the product of interest (i.e., SST knockout). Consequently, the current study focused on the identification of potential new sst5 variants in different rodent models, and their characterization under normal or pathophysiological states. The results obtained provide the first evidence for the existence of three truncated but functional sst5 variants in mouse (named sst5TMD4, sst5TMD2 and sst5TMD1) and one in rat (named sst5TMD1). These receptors, which show high inter-specific sequence identity, unique tissue expression patterns and distinct ligand-selective signaling properties, may help to better understand the complex pathophysiology of the SST/CST/sst system.

Materials and methods

Reagents

Unless indicated otherwise, all chemical products and tissue culture reagents were purchased from Sigma–Aldrich. D-MEM was obtained from Cambrex (Milan, Italy), fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria), tissue culture plasticware was obtained from TPP (Trasadingen, Switzerland), mouse/rat somatostatin-14 (SST) and cortistatin-14 (CST) were purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany).

Animal models

All experimental procedures were approved by the Animal Care and Use Committees of the University of Cordoba, University of Illinois at Chicago and the Jesse Brown VA Medical Center. Male and female C57Bl/6 J, Swiss and FVB mice and Wistar rats were purchased from “Centro de Instrumentación Científica” (University of Granada, Spain) or from Jackson Laboratories (Bar Harbor, ME). ob/ob mice and their lean littermate controls were purchased from Jackson Laboratories. SST-KO mice were provided by Dr. Ute Hochgeschwender [27]. MT-GHRH mice were provided by Dr. Robert E. Hammer [28]. All animals were housed under standard conditions of light (12-h light, 12-h

dark cycle; lights on at 0700 h) and temperature (22–24°C), with free access to tap water and food (standard rodent chow; LabDiet, Framingham, MA). All animals were handled daily at least 1 week prior to euthanasia to acclimate to personnel and handling procedures. Animals were killed by decapitation, without anesthesia, under fed conditions unless otherwise specified. Trunk blood was collected for hormone and metabolite determinations, and fresh tissues were collected for primary tissue cell culture or were frozen in liquid nitrogen and stored at −80°C until their further analysis of mRNA levels by conventional or qrtRT–PCR (see below). Descriptions of the experiments conducted follows:

Distribution of sst5 variants in mouse tissues

Expression of sst5 variants was screened by qrtRT-PCR in a variety of mouse tissues using specific primers for each transcript (Table 1; primer sets 7–10).

Effect of fasting on the hypothalamus (HPT) and pituitary (PIT) expression of mouse sst5 variants

To evaluate the effect of fasting on sst5 variants expression, male mice (10 weeks of age) were assigned to one of four groups ($n = 5$–8): 12, 24, 48 h fasting or control feeding ad libitum and HPT and PIT were collected for further analysis as previously reported [29].

Effect of obesity on the HPT and PIT expression of mouse sst5 variants

To study the effect of the obese state on sst5 variant expression, tissue samples were obtained from two models of obesity as previously reported [30, 31]: (1) ob/ob mice and their littermate controls ($n = 6$; 10 weeks of age); (2) diet-induced obesity (DIO) mice, fed a low-fat diet (LFD) or a high-fat diet (HFD) diet. Briefly, DIO groups of male mice ($n = 6$–7) were placed at 4 weeks of age on either a LFD (10% kcal from fat, used as control; Research Diets, Brunswick, NJ) or HFD (60% kcal from fat) and killed at 20 weeks of age.

Effect of leptin on the HPT and PIT expression of mouse sst5 variants

To evaluate the impact of leptin replacement on sst5 variants expression in the ob/ob mouse, HPT and PIT from ob/ob male mice (10 weeks of age) used in a recent study [30] were further evaluated. Briefly, groups of ob/ob mice ($n = 5$) were implanted with osmotic mini-pumps (sc) containing either recombinant mouse leptin or vehicle for 7 days. This study included a group of ob/ob mice pair-fed to match the food intake of leptin-treated mice to differentiate between direct effects of leptin and those mediated indirectly by leptin-induced reduction in food intake and weight loss.

Expression of sst5 variants on the HPT and PIT of SST-KO mice

To explore the potential role of endogenous SST in the regulation of sst5 variant expression, tissues samples from male SST-knockout (SST−/−) and SST-intact (SST+/+) mice were used as previously reported [32, 33]. Briefly, SST+/+ and SST−/− mice ($n = 5$–8 per group; 9–11 weeks old) were fed ad libitum or subjected to food deprivation for 48 h. After this period, mice were killed by decapitation and tissues collected for analysis.

Expression of mouse sst5 variants on PIT tumors

qrtRT-PCR was used to determine whether sst5 variants were present in hyperplastic and adenomatous PIT samples from male mice expressing the MT-hGHRH transgene and normal PIT samples of age-matched wild-type controls previously generated by our laboratory [34].

RNA isolation and reverse transcription (RT)

Tissues and cell cultures were processed for recovery of total RNA using the Absolutely RNA RT–PCR Miniprep Kit (Stratagene, La Jolla, CA) or Trizol reagent (Invitrogen, Barcelona, Spain) with deoxyribonuclease treatment (Stratagene; Promega, Madison, WI) as previously described [31, 32, 35]. The amount of RNA recovered was determined using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1–2 μg for whole tissue extract and 0.25 μg for primary cell cultures) was reversed transcribed with enzyme and buffers supplied in the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNA was treated with ribonuclease H (1U; MRI Fermentas).

Isolation and sequence of the sst5 variants by standard RT-PCR

Initial amplification and sequencing of truncated sst5 variant transcripts were performed by standard RT-PCR, named type I, using tissue extracts (HPT and PIT) from adult C57Bl/6 J, Swiss and FVB mice and Wistar rats. Primers used for standard PCR type I (Table 1; Primer sets 1–2) were located upstream of the start codon (sense primer: msst5-5′UTR and rsst5-5′UTR) and downstream of the stop codon (antisense primer: msst5-3′UTR and rsst5-3′UTR) of the mouse and rat sst5 gene (Genbank accession

Table 1 Specific primers used for amplification of the different mouse and rat sst5 transcripts (msst5 and rsst5, respectively) in conventional RT-PCR (RT-PCR I and II) and quantitative real-time RT-PCR (RT-PCR III)

Set no.	Name	Primer sequence	Nucleotide position	Product size (bp)	Tm (°C)
RT-PCR I primers					
1	msst5-5′UTR	Sn: GGCTCCTCTAACATTGTCT	260	1,844	58
	msst5-3′UTR	As: GTCCATTCTCTTTCCAGTCTT	2,104		
2	rsst5-5′UTR	Sn: GCACCCTGTCCTGCACAGAGACACG	97	1,268	64
	rsst5-3′UTR	As: ACACTGCCACCGAGAGGAGTCCAAC	1,364		
RT-PCR II primers					
3	msst5-nested5′a	Sn: GGTCATCTATGTGGTGTTGCGGT	491	1,617	62
	msst5-nested3′a	As: GTGTGTCCATTCTCTTTCCAGTCTT	2,108		
4	msst5-nested5′b	Sn: CTATGTGGTGTTGCGGTACG	497	1,398	60
	msst5-nested3′b	As: GGGCTGAACACACATGGATACCTTT	1,895		
5	msst5-nested5′c	Sn: CGGTATTAGTGCCTGTGCTCTACTT	429	1,045	62
	msst5-nested3′c	As: CTCTCATATCCCAGAAGACAACACC	1,474		
6	rsst5-nested5′	Sn: CGGCTGCTTCCAGTGGTAACCATA	197	1,164	64
	rsst5-nested3′	As: TGCCACCGAGAGGAGTCCAACTTGT	1,360		
RT-PCR III (qrtRT-PCR) primers					
7	Qrt-msst5	Sn: ACCCCCTGCTCTATGGCTTT	1,215	105	61
		As: GCTCTATGGCATCTGCATCCT	1,319		
8	Qrt-msst5TMD4	Sn: GTCCACCCTCTCCGCTCA	415	131	61
		As: GCAGGTTCGCAGAGGACATC	545		
9	Qrt-msst5TMD2	Sn: CAGTTCACCCGTACTGTGGCAT	358	132	61
		As: CACAGCTTCAGGGTGGGTAA	489		
10	Qrt-msst5TMD1	Sn: AACGTGTATATCCAGACAAGAGTGG	217	152	61
		As: TCCCAGAAGACAACACCACA	368		
11	Qrt-rsst5	Sn: GCTCATGTCTCTGCCGCTCTT	648	281	61
		As: AGCCCACAAACACCAGCACCACGAC	928		
12	Qrt-rsst5TMD1	Sn:ACGCCAAGATGAAGACAAGAGT	194	154	61
		As:CAGAACCCAGAAGACAACACC	347		

Set no.	Name	Primer Sequence	Products size (bp)	Tm (°C)
Primers to clone msst5 variants in pcDNA3.1				
13	Se_Start codon	Sn: ATGGAGCCCCTCTCTTTGAC	1,716 (msst5); 668 (msst5TMD4)	61
	As_3′UTR	As: CCTTGCTTTTCCTTGTGCTG	713 (msst5TMD2); 938 (msst5TMD1)	
14	Sense_EcoRI_Kozak	Sn: TAATGAATTCCCACCATGGAGCCCCTCTC	1,187 (msst5)	61
	Antisense_XhoI_msst5/TMD1	As: TAATCTCGAGTCAGCAAACTCTCAT	406 (msst5TMD1)	
15	Sense_EcoRI_Kozak	Sn: TAATGAATTCCCACCATGGAGCCCCTCTC	601 (msst5TMD4)	61
	Antisense_XhoI_ msst5TMD4/2	As: TAATCTCGAGTCAGCAAACTCTCAT	646 (msst5TMD2)	
Primers to clone 3xHA-tagged-msst5 variants in pcDNA3.1				
16	Sense_HindIII_Kozak	Sn: ACTTAAGCTTGGTACCACCATGTAC	117	61
	Antisense_EcoRI	As: TAATGAATTCCATCAGCGTAATCTG		
17	Sense_EcoRI	Sn:TAATGAATTCTTATGGAGCCCCTCTC	1,184 (msst5)	61
	Antisense_XhoI_ msst5/TMD1	As: TAATCTCGAGTCAGCAAACTCTCAT	403 (msst5TMD1)	
18	Sense_EcoRI	Sn:TAATGAATTCTTATGGAGCCCCTCTC	598 (msst5TMD4)	61
	Antisense_XhoI_ msst5TMD4/2	As: TAATCTCGAGTCAGCAAACTCTCAT	643 (msst5TMD2)	

GenBank accession numbers used were NM_011425.1, GQ359775, GQ359776, GQ359777, NM_012882 and GQ359778 for full-length sst5, sst5TMD4, sst5TMD2, sst5TMD1 (mouse variants) and full-length sst5 and sst5TMD1 (rat variants), respectively

bp base pairs, *Sn* sense, *As* antisense

no. NM_011425.1 and NM_012882, respectively). PCR reactions (type I) were performed using the iCycler IQTM system (BioRad, Madrid, Spain), 1.25U EcoTaq polymerase (Ecogen, Barcelona, Spain), 200 μM dNTPs, 2 mM $MgCl_2$ (Ecogen), 0.2 μM of primers, 1 M betaine and 100 ng of cDNA as template in a 25 μl-volume reaction with a program consisting of the following steps: (1) 95°C for 10 min, (2) 38 cycles of denaturation (95°C for 30 s), annealing (58–64°C for 30 s) and extension (72°C for 30–120 s), and (3) final extension of 10 min at 72°C. All PCR products were run on agarose gel, stained with ethidium bromide and column-purified using QuiaQuick gel extraction kit (Qiagen, GmBH, Germany). All PCR products were sequenced to confirm target specificity at the Genomic Unity of the University of Cordoba using an ABI Prism 3130XL Genetic analyzer (Applied Biosystems, Madrid, Spain). Bioinformatics analyses of the isolated fragments were performed to blast the sequences and deeply analyze the novel spliced sst5 transcripts. Then, a second nested PCR (named type II) was carried out to re-amplify single PCR products with nested primer pairs (Table 1; primer sets 3–6) located more internally in the mouse and rat sst5 gene than the primers used in PCR type I.

Verification and quantification of the truncated sst5 variant expression using qrtRT–PCR

Primers used for standard and qrtRT–PCR (Table 1; primers sets 7–12) were selected using Oligo 6.0 (Molecular Biology Insights, Inc., Cascade, CO) and Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) software [36]. Details regarding the development, validation and application of a qrtRT-PCR to measure expression levels of mouse transcripts, including full-length sst5, have been reported previously [29, 31, 32]. Similarly, sets of primers were designed and validated to specifically amplify and quantify the truncated sst5TMD4/2/1 variants in different RT-PCR samples. Specifically, to determine the starting copy number of cDNA, RT samples were PCR amplified, and the signal was compared with that of a standard curve run on the same plate. Specific standard curves consisted of 1, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of synthetic cDNA template for each of the transcripts of interest (full-length and truncated sst5 variants) were constructed. In addition, total RNA samples that were not reversed transcribed and a no DNA control were run on each plate to control for genomic DNA contamination and to monitor potential exogenous contamination, respectively. Also, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, the mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (used as housekeeping gene), where cyclophilin A mRNA levels did not significantly vary between experimental groups within tissue type (data not shown).

Cloning, epitope-tagging and transfection of mouse sst5 variants

cDNA sequences encoding the whole open reading frame (ORF) of all mouse sst5 variants (full-length and truncated sst5) were generated by a RT-PCR strategy using mouse RNA (pituitary and fat) as a template, the iMAX-II polymerase kit (iNtRON Biotechnology, Seongnam, Korea) and 0.2 μM of primers (Table 1; primer sets 13–18). The PCR products were amplified and purified from the gel and then fragments were sequenced at least three times to verify the correct ORF. These fragments were subsequently used for two sets of studies:

Functional studies

PCR fragments of the sst5 variants were introduced into the pCDNA3.1 vector using specific primers containing a Kozak sequence and a EcoRI site (sense primer) and a XhoI site (antisense primer) (Table1; primer sets 14–15) and then stable transfected into CHO-K1 cells as previously described [26, 37]. Cells stable transfected with each sst5 variant were used to evaluate the modulation of free cytosolic $[Ca^{2+}]_i$ in single cells by microfluorimetry and the measurement of cAMP levels in response SST-14 and CST-14 (see below).

Cellular localization studies

PCR fragments of the sst5 variants were introduced into pCDNA3.1 vector using specific primers containing EcoRI and XhoI sites (Table1; primer sets 17–18), and a 3xHA tag was synthesized (Table 1; primer set 16), self-annealed and inserted (in phase upstream of the sst5 variant fragments) into the HindIII and EcoRI sites acquiring the different mouse 3xHA-tag-sst5 expressing vectors. The triple ligation of all sst5 variants was validated by PCR sequence. These 3xHA-tag-sst5 vectors were transiently transfected into CHO-K1 cells as previously described [37], and then cells were used for confocal localization assays (see below).

In both sets of studies, the cloning and transfection of sst5 variants were performed employing the reagents and methods reported previously [26, 37]. Expression analysis validations in CHO-K1 cells transfected with each sst5 variant by qrtRT-PCR showed an equally high transfection efficiency level (data not shown).

Cell culture models

CHO-K1 cells

Transient and stable cells transfected with sst5 variants were cultured as previously described [37].

Primary PIT cell and HPT N6 cell cultures

PIT of adult male C57Bl/6 J mice (10 weeks of age) were dispersed into single cells and cultured in serum containing α-medium, as previously described [30, 31]. N6 cells, a mouse HPT cell line originally developed by Belsham et al. [38], were cultured in monolayer in serum containing α-medium, as previously described [39, 40]. After 24 h of culture (200,000 cells/well, 24-well plates), media was removed, and wells were washed in serum free media and subsequently treated with SST-14 and CST-14 (100 nM) for 24 h, and then total cellular RNA was extracted for determination of sst5 variant expression by qrtRT–PCR.

Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells

CHO-K1 cells stably transfected with each of the sst5 variants were grown onto 25 mm-Ø glass coverslips for 24 h (50,000 cells/coverslip, 35-mm plates) and incubated for 30 min at 37°C with 2.5 μM of the Ca^{2+} indicator dye Fura-2 AM (Molecular Probes) in phenol red-free DMEM containing 20 mM $NaHCO_3$ (pH 7.4). Coverslips were washed with phenol red-free DMEM, mounted on the stage of a Nikon Eclipse TE200-E microscope (Nikon, Tokyo, Japan) with an attached back thinned-CCD cooled digital camera (ORCAII BT; Hamamatsu Photonics, Hamamatsu, Japan). Cells were examined under a ×40 oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured every 5 s as previously reported [35, 37]. Changes in $[Ca^{2+}]_i$ after SST-14 and CST-14 (100 nM) administration were recorded as background substrate ratios of the corresponding excitation wavelength (F340/F380) using MetaFluor Software (Imaging Corp., West Chester, PA).

cAMP measurements

To investigate the responsiveness of CHO-K1 cells stably transfected with each of the sst5 variants or transfected with the empty vector (used as a control), cells were plated (10,000 cells/well, 96-well plates) and cultured in serum containing F12 media. After 24 h of culture, media was removed and cells washed in serum-free media for 40 min, and then preincubated with F12 containing 1 nM of 3-isobutyl-1-methylxanthine (IBMX) to prevent enzymatic degradation of cAMP. After 30 min of preincubation with IBMX, cells were incubated for an additional 30-min period with control (media), SST-14 or CST-14 (100 nM) in the presence of IBMX and 100 nM of forskolin to evaluate the ability of SST and CST to inhibit forskolin-stimulated cAMP production. The experiment was terminated by adding lysis reagent to each well, and lysates were recovered and stored at −80°C for analysis of intracellular cAMP accumulation, as assessed by cAMP Biotrack EIA system following Protocol 3 of the manufacturer's instructions (GE Healthcare, Barcelona, Spain).

Subcellular localization and confocal microscopy

Transiently transfected CHO-K1 cells with each sst5 variant plasmids containing a 3xHA epitope were cultured on round 18-mm Ø glass coverslips (50,000 cells/well, 12-well plates) in serum containing media, as previously described [37]. After 24 h of culture at 37°C, media was removed and cells washed in serum free media and fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were rinsed sequentially with PBS (three times, 5 min each), permeabilized with 50% metanol-PBS (3 min at −20°C) and 100% metanol (3 min at −20°C), blocked with PBS-0.5% BSA (2 h at 4°C) and then incubated overnight (4°C) with primary antibody for 3xHA epitope (1:1,000; self-made) [26]. Finally, cells were incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; 1:500 in PBS; 2 h at 4°C; Invitrogen, Eugene, OR), washed in PBS and mounted with glycerol PBS. The signal was visualized using a Leica Espectral TCS-SP2-AOBS confocal scanning microscope (Leica Corp., Heidelberg, Germany) and then analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Controls consisted of transiently transfected CHO-K1 cell preparations processed as described above with the exception of excluding primary antibody to 3xHA epitope.

Statistical analysis

Samples from all groups within an experiment were processed at the same time; therefore, the in vivo effects of genotype/fasting/obesity and the in vitro effects of SST/CST were assessed by one- or two-way ANOVA followed by a Newman-Keuls test for multiple comparisons or by Student's t test, as appropriate. $P < 0.05$ was considered significant. All data are expressed as means ± SEM. The in vivo effects of genotype/fasting/obesity were obtained from a minimum of five animals per group. Results from in vitro studies were obtained from at least three separate, independent experiments carried out on different days and with different cell preparations. For single cell analysis, a

minimum of 20 cells were analyzed per experiment. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD).

GenBank submission

The new sequence of the mouse and rat sst5 variants were submitted to Genbank (accession nos. GQ359775, GQ359776, GQ359777 and GQ359778 for the mouse sst5TMD4, sst5TMD2, sst5TMD1 and rat sst5TMD1 variants, respectively).

Results

Identification of mouse and rat sst5 variants using standard RT–PCR

Three specific bands in mouse tissues and one band in rat tissues corresponding to novel variants of the sst5 gene were obtained by standard RT-PCR (type I and II, see "Materials and methods" section) using primers specified in Table 1. Each sst5 variant was separately amplified by RT-PCR (type III) using selective-sst5 variant primers (see below). All PCR products did not appear using total RNA samples that were not reversed transcribed or in PCR that did not contain samples, indicating that they were not the result of genomic contamination or potential exogenous contamination, respectively. cDNA sequencing and bioinformatic analysis of the isolated fragments revealed that these products have a complete identity with discontinuous segments of the mouse and rat sst5 gene with full identity against two different and distant areas of the sst5 genomic sequence [GeneID nos. 20609 (mouse) and 25354 (rat); Fig. 1a and supplemental Figs. 1a, 2a]. The new truncated sst5 variants described herein are not generated by a classical alternative splicing mechanism since the sst5 gene lacks canonical introns within its coding sequence (CDS), which is fully encoded within the exon-3 (mouse) or the exon-2 (rat) of the sst5 genomic sequences published to date in the NCBI website (GeneID nos. 20609 and 25354, respectively). Thus, the generation of new truncated sst5 variants should involve the presence of cryptic introns with non-canonical donor and acceptor splice sites within the CDS and the 3′UTR region, resulting in new receptors that maintain the same N-terminal region (CDS-1; Fig. 1b–d, left panel) as full-length sst5 isoform (Fig. 1a, left panel), but have different, shorter C-terminal tails (CDS-2; Fig. 1b–d, left panel) with 4 (mouse sst5TMD4), 2 (mouse sst5TMD2) and 1 (mouse and rat sst5TMD1) TMD (Fig. 1b–d, middle and right panels and supplemental Fig. 1a, 2a, b). Therefore, assuming that the start of the transcription is unaltered in the novel sst5 variants, the complete CDS of 576, 621 and 384 nucleotides (for the mouse sst5TMD4, sst5TMD2 and sst5TMD1 variants, respectively), and of 348 nucleotides (for the rat sst5TMD1 variant) would encode proteins of 191, 206, 127 and 115 amino acids, respectively (Supplemental Fig. 1a, 2). It is interesting to note that the mouse and rat sst5TMD1 variants showed high interspecific nucleotide (87%) and amino acid (86%) sequence identity (Supplemental Fig. 2c–d).

Verification and quantification of the mouse sst5 variant expression in different tissues using qrtRT PCR

Our aim was to accurately compare the level of expression of the new truncated sst5 variants with that of full-length sst5 in various tissues. Therefore, sets of primers were designed and validated to amplify specifically the truncated mouse sst5TMD4, 2 and 1 variants by qrtRT–PCR. To this end, one of the primers was designed to span the two partial coding regions (CDS-1/CDS-2) of the different truncated sst5 variants [on top of the joining site (open arrows in Fig. 2a)]. Using these new sets of primers and the one previously validated to amplify sst5 [29], a single PCR product was consistently obtained, which was found to be of the expected size (105 bp for full-length sst5, 131 bp for sst5TMD4, 132 bp for sst5TMD2 and 152 bp for sst5TMD1 by separation in agarose gel; Fig. 2b) in all tissues analyzed. The temperature of dissociation of these PCR products differed (Fig. 2c), indicating that distinct products were generated as confirmed by the sequencing analysis. Analysis of the expression pattern of the sst5 variants in normal tissues shows that full-length and truncated sst5 variants are widely and differentially expressed in the mouse tissues tested (Fig. 2d and supplemental Fig. 3). Specifically, we found that sst5 was predominantly expressed in the pituitary (PIT) and hypothalamus (HPT) (21,163 ± 4,343 and 4,638 ± 473 copies, respectively; Fig. 2d). Similarly, sst5TMD2 and sst5TMD1 were highly expressed at the PIT and HPT level compared with other tissues (PIT: 15,185 ± 3,437 and 1,044 ± 207 copies; HPT: 2,950 ± 490 and 197 ± 35 copies, respectively). In contrast, the expression level of sst5TMD4 variant was very low in those tissues (PIT: 15 ± 2 and HPT: 5 ± 2). Analysis of the other tissues revealed a heterogeneous pattern of expression depending on the sst5 variant and the tissue screened. Thus, sst5 and sst5TMD2 are the most represented variants at the systemic level, whereas sst5TMD4 is scarcely expressed, being only detectable in the gut, ovary, uterus, fat and mammary gland, and sst5TMD1 was preferentially found in heart and at low levels in other tissues where sst5 and sst5TMD2 are expressed (Fig. 2d and supplemental Fig. 3).

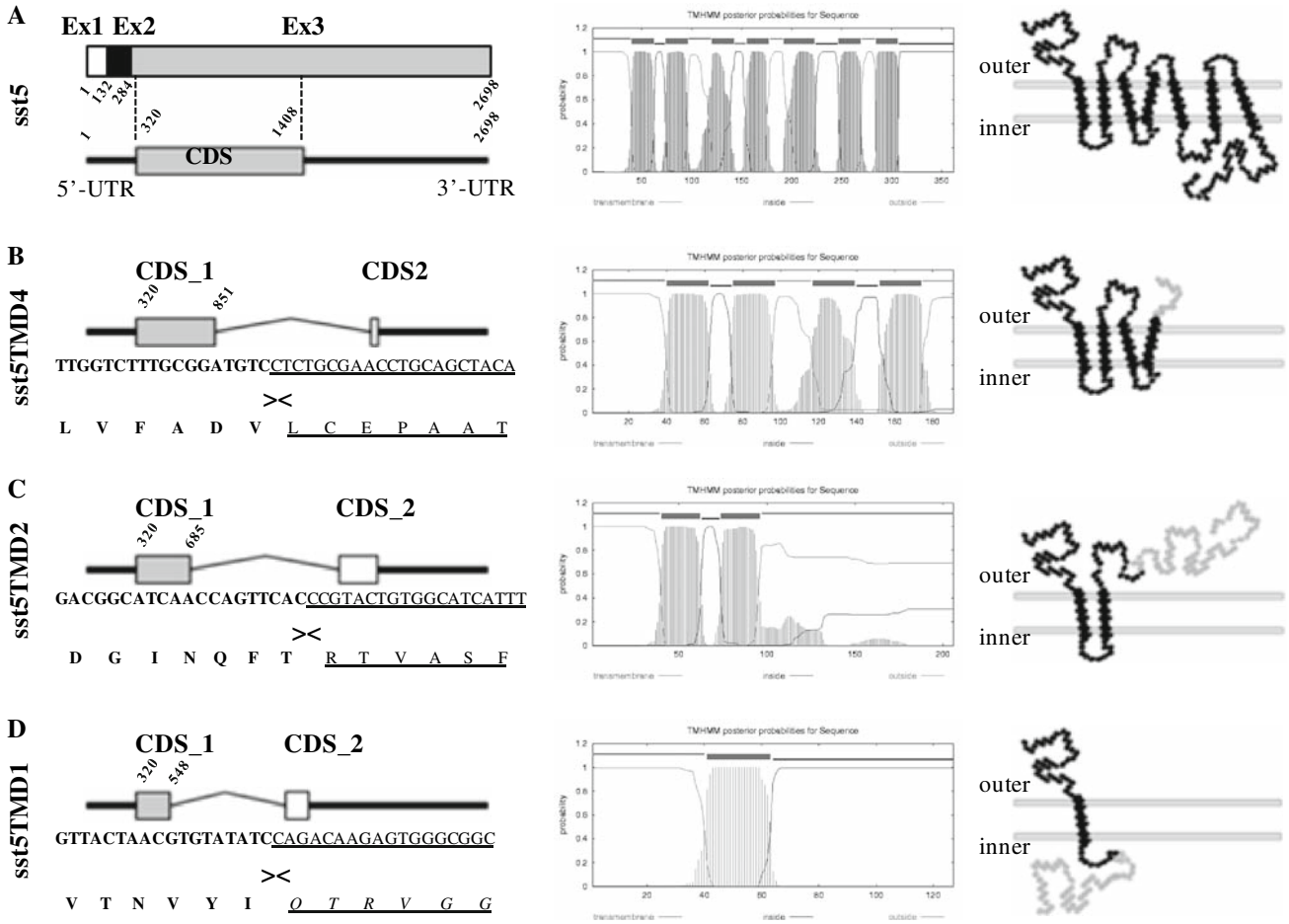

Fig. 1 Molecular identification and characterization of novel truncated mouse sst5 variants. **a** *Left panel* schematic representation of the full-length sst5 mRNA constituted by three exons: exon 1 (Ex1; *white box*; nucleotide nos. 1–132), exon 2 (Ex2; *black box*: nucleotide nos. 133–284) and exon 3 (Ex3; *grey box*; nucleotide nos. 285–2698). Coding region (CDS) is located and codified within the Ex3 (nucleotide nos. 320–1408). **b, c** *Left panel,* schematic representation of the truncated sst5 variant mRNA constituted by the same 5′UTR sequence (*straight black line* on the left) as full-length sst5 (including Ex1, Ex2 and first 34 bp of Ex3), two coding regions (CDS_1 and CDS_2) and a 3′UTR sequence (*straight black line* on the right). The N-terminal regions of the CDS_1 (*grey boxes*) are similar in all sst5 variants (full-length and truncated) but have different length (nucleotide nos. 320–857 in sst5TMD4, 320–685 in sst5TMD2 and 320–548 in sst5TMD1), while CDS_2 of each truncated sst5 variant (*white boxes*) is constituted by unique sequences originating by splicing of a portion of the CDS and/or 3′-UTR of the full-length sst5. The nucleotide and amino-acid sequences of the junction between the CDS_1 and CDS_2 of the different truncated sst5 variants are shown below the corresponding schematic representation of each sst5 variant. **a–d** *Middle panel* hydrophobicity profiles of full-length and truncated sst5 variants obtained from the program TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) (CBS, Denmark) and *right panel* predicted transmembrane domain structure of full-length and truncated sst5 variants. In grey are shown the different C-terminal tails of each truncated sst5 variant compared with full-length sst5 variant

Subcellular localization of mouse sst5 variants in Chinese hamster ovary (CHO)-K1 cells

Analysis of the subcellular localization of each of the sst5 variants (3xHA-tagged receptors; Fig. 3) in CHO-K1 cells by confocal microscopy revealed that whereas the full-length sst5 variant is preferentially located at the plasma membrane (Fig. 3a), the truncated sst5 variants are mainly located at intracellular compartments (Fig. 3b–d). Nonetheless, a proportion of all of the truncated sst5 variants was also located at the plasma membrane (Fig. 3b–d).

Measurement of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

The ability of each sst5 variant to modify the $[Ca^{2+}]_i$ kinetics in response to their natural ligands, SST and CST, was evaluated in CHO-K1 cells stably transfected with full-length sst5, sst5TMD4, sst5TMD2 or sst5TMD1 (Fig. 4a, b). This approach demonstrated that cells transfected with sst5 responded to SST and CST in a similar manner in terms of the proportion of responsive cells (47 and 40% respectively) and the profile of the response evoked, which showed

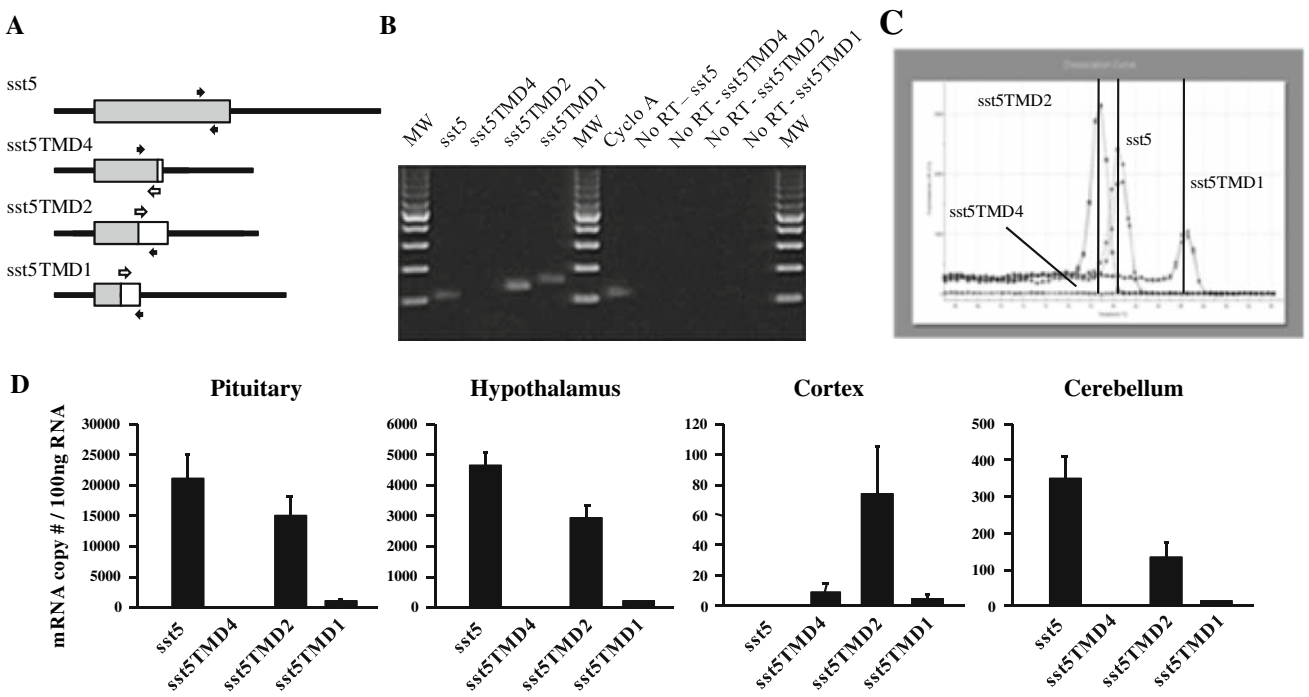

Fig. 2 Verification and quantification of the full-length and truncated mouse sst5 variants in mouse tissues. **a** Schematic representation of the full-length sst5 and novel truncated sst5 variant transcripts. CDS are shown by boxes (*grey boxes* CDS of sst5 or CDS_1 of each truncated sst5 variant; *white boxes* CDS_2 of each truncated sst5 variant). *Arrowheads* are used to indicate the relative positions of primers sets used in quantitative real-time RT-PCR (qrtRT-PCR; closed arrows indicated primers within a CDS, while open arrows indicate primers spanning CDS_1 and CDS_2). **b** Representative agarose gel showing PCR products amplified by qrtRT-PCR using mouse pituitary as template. All products (sst5 variants and cyclophilin used as a housekeeping gene) were size separated on an agarose gel containing EtBr, column-purified and sequenced to confirm target specificity. PCR product of the sst5TMD4 variant could not be amplified if pituitary cDNA was used as a template, indicating that this variant was not present in this tissue; however, the sst5TMD4 variant transcript was detected in other tissues (i.e., fat; Supplemental Figure 3). **c** Melting curves of qrtRT-PCR products with different T_m of dissociation (80.9°C for full-length sst5, 79.4°C for sst5TMD2 and 86.8°C for sst5TMD1) generated from the same mouse pituitary sample. **d** Absolute cDNA copy number/0.1 µg total RNA of full-length and truncated sst5 variant transcripts in the pituitary, hypothalamus, cortex and cerebellum of male C57Bl/6 mice, as determined by qrtRT-PCR. Values represent means ± SEM ($n = 5$ mice)

comparable temporal and quantitative features (Fig. 4a). Similar to sst5, cells transfected with sst5TMD1 showed a comparable, albeit lower proportion of cells responsive to SST and CST (20 and 11%, respectively). In striking contrast, cells transfected with sst5TMD4 responded almost exclusively (48%) to SST, since only 2% of those cells responded to CST. Interestingly, an opposite situation was observed for sst5TMD2, in that only 13% of the cells responded to SST by showing delayed responses (28 s), whereas CST evoked rapid and clear $[Ca^{2+}]_i$ increases in 51% of the cells transfected with this receptor. SST or CST treatment did not evoke any changes in $[Ca^{2+}]_i$ in non-transfected CHO-K1 cells or in cells transfected with the empty vector (used as controls; data not shown).

cAMP measurement

To further analyze the functionality of the truncated sst5 variants, we examined the ability of SST and CST to modify forskolin-stimulated cAMP production in transfected CHO-K1 cells. As shown in Fig. 4c, SST significantly diminished cAMP production in cells expressing sst5TMD2 or sst5TMD1 (20% and 40% respectively compared with control). In marked contrast, we observed a trend toward an opposite, stimulatory effect of SST in cells transfected with full-length sst5 or with truncated sst5TMD4 (45% and 36%, respectively, compared with control), although these differences did not reach statistical significance. On the other hand, CST did not cause any significant effect in cells transfected with full-length or truncated sst5 variants (Fig. 4c).

Metabolic regulation of the HPT and PIT expression of mouse sst5 variants

Given the fact that the PIT and HPT full-length sst5 mRNA levels can be regulated under different states of energy balance and by different metabolic signals [2, 29], we sought to study the PIT and HPT transcriptional regulation of the new truncated sst5 variants in a series of mice

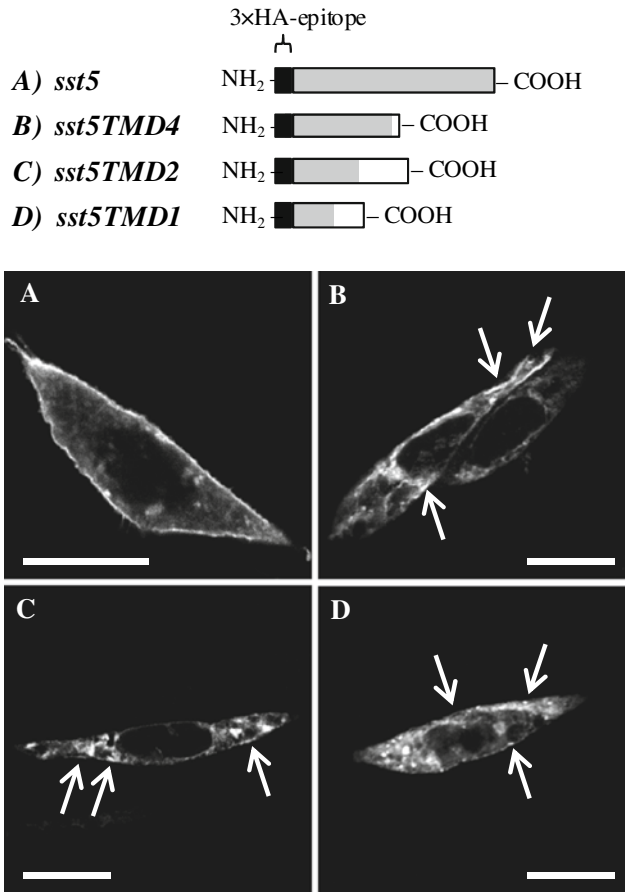

Fig. 3 Subcellular localization of full-length and truncated mouse sst5 variants. *Top panel* Schematic representation of the recombinant protein of full-length (**a**) and truncated sst5 variants [sst5TMD4 (**b**), sst5TMD2 (**c**) and sst5TMD1 (**d**)] containing the 3x hemagglutinin (3xHA) epitope in the N-terminal domain. *Black boxes* represent 3xHA epitope, and grey and white boxes represent CDS_1 and CDS_2, respectively. *Bottom panel* Representative images of the subcellular localization of the full-length (**a**) and truncated sst5 variants [sst5TMD4 (**b**), sst5TMD2 (**c**) and sst5TMD1 (**d**)] transfected in CHO-K1 cells using confocal microscopy. *White arrows* indicate regions of plasma membrane where truncated sst5 variants are visualized. *Scale bar* 10 μm

models under different metabolic/pathologic conditions previously characterized in our laboratory, which include: fasting, diet-induced obesity (DIO), obese mice due to leptin-deficiency (ob/ob), mice expressing the metallothionein promoter-human GHRH transgene (MT-hGHRH) and somatostatin knockout mice (SST-KO) [29–33] (Fig. 5a and Supplemental Figure 4). In addition, changes in the expression levels of all sst5 variants were evaluated in primary PIT cell cultures as well as in the murine hypothalamic cell line N6 [38] in response to treatments with endogenous ligands for these receptors (SST and CST; Fig. 5b, c, respectively). It should be noted that, as mentioned above, expression levels of sst5TMD4 were not detected or were extremely low in the normal mouse PIT and HPT; therefore, the analysis of this variant was not included in the corresponding figures.

Effect of negative energy balance conditions (fasting time-course)

Fasting evoked a significant marked decrease in the expression levels of all sst5 variants in the PIT, but did not significantly alter HPT mRNA levels (Supplemental Fig. 4a)

Effect of conditions of positive energy balance (obesity)

No significant differences were observed in expression levels of sst5 variants in PIT or HPT between obese mice fed on a high-fat diet (HFD) as compared with mice fed on a low-fat diet (LFD) used as control (Supplemental Fig. 4b). Likewise, no differences were found between ob/ob mice and lean control animals (Supplemental Fig. 4c). Moreover, when the effect of leptin replacement on the PIT and HPT expression of sst5 variants was analyzed in ob/ob mice, we found that leptin infusion did not significantly alter the expression levels of PIT and HPT sst5 variants (vehicle control ob/ob compared with leptin-treated ob/ob and pair-fed ob/ob mice; Supplemental Fig. 4d).

Presence of sst5 variants in PIT tumors (MT-hGHRH transgenic mice)

We have previously observed that the full-length sst5 variant is highly expressed in PIT tumors of MT-GHRH mice and that the expression levels of this receptor are upregulated in the hyperplastic PIT (4 month-old) compared to age-matched controls, while adenoma formation (mice >10 months of age) is associated with a decline in sst5 expression compared to WT levels (Supplemental Fig. 4e, left panel) [34]. Therefore, we sought to determine if the novel truncated sst5 variants were also present in mouse PIT tumors and whether the expression levels of these variants were regulated in the hyperplastic and adenomatous state. Our results demonstrate that, similar to that found in normal pituitaries, sst5TMD2 and sst5TMD1 are present in both hyperplastic and adenomatous PIT tumors of MT-GHRH mice. In striking contrast to that found for full-length sst5, we found that expression levels of sst5TMD2 or sst5TMD1 were not altered in hyperplastic or adenomatous pituitaries compared with their respective controls (Supplemental Fig. 4e, middle and right panels).

Effect of lack of SST (SST-KO mice)

Given the essential role of SST in regulating the expression levels of its own receptors [2], we also sought to determine

A

	SST-14			CST-14		
	% cells	*% Max ± SEM*	*Time ± SEM*	*% cells*	*% Max ± SEM*	*Time ± SEM*
sst5	47	359,8 ± 23,0	15,4 ± 0,7	40	316,0 ± 21,3	14,8 ± 1,4
sst5TMD4	48	313,9 ± 19,3	23,1 ± 1,6	2	341,1 ± 121,0	22,5 ± 4,8
sst5TMD2	13	269,9 ± 12,9	28,4 ± 1,8	51	254,3 ± 4,9	19,9 ± 0,8
sst5TMD1	20	290,9 ± 4,9	18,8 ± 0,2	11	230,5 ± 33,2	22,2 ± 2,6

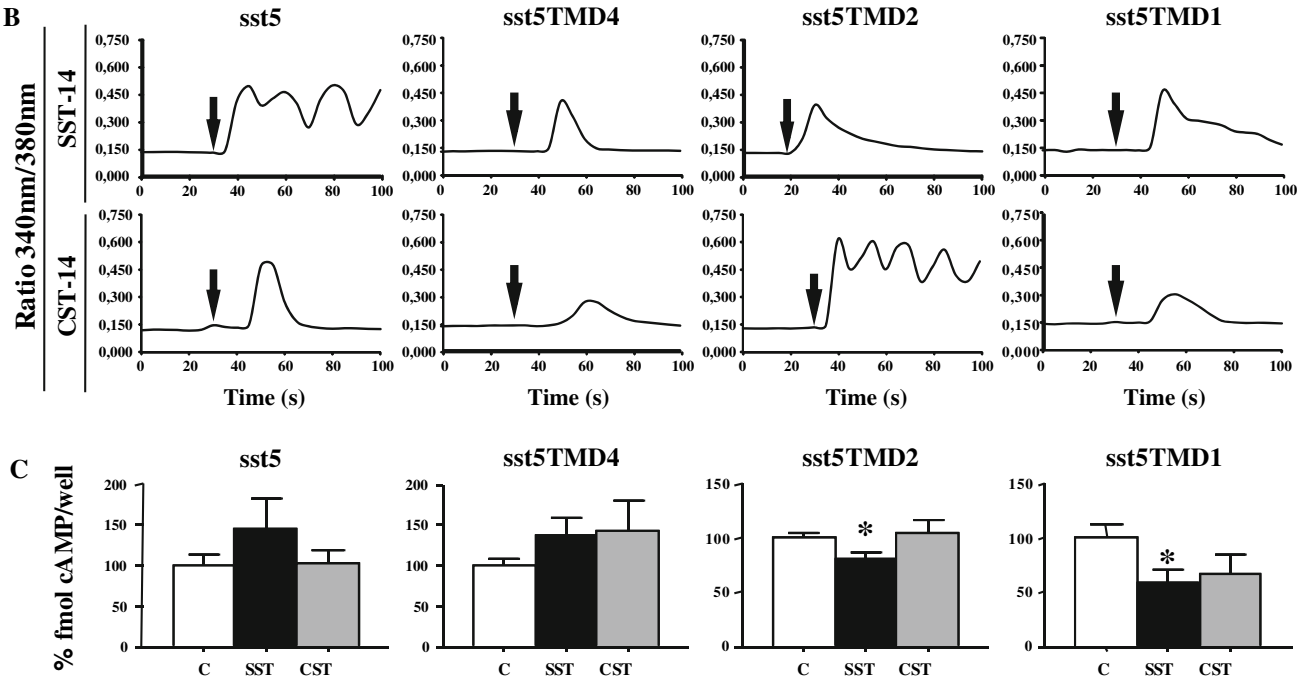

Fig. 4 Functional characterization of full-length sst5 and truncated variants in terms of activation of signal transduction ($[Ca^{2+}]_i$ and cAMP) in response to somatostatin (*SST*) and cortistatin (*CST*). CHO-K1 cells were stable-transfected with full-length or truncated sst5 variants, and the effect of SST and CST challenge (100 nM) was evaluated. **a** Percentage of transfected cells showing changes in $[Ca^{2+}]_i$ in response to SST and CST. Percentage of maximum response (*%Max*) and time of response to SST and CST administration are also indicated. **b** Representative profiles of changes in $[Ca^{2+}]_i$ in transfected cells in response to SST and CST administration (*arrow*). **c** Effect of SST and CST treatment on the forskolin-induced cAMP production in transfected cells (*C* control, $n = 4$ experiments). *Asterisk* ($*P < 0.05$) indicates values that differ from control-treated cells

the potential function of SST as a modulator of the expression levels of the new truncated sst5 variants. To this end, PIT and HPT of SST-KO mice were analyzed. We observed that lack of endogenous SST did not modify PIT or HPT mRNA levels of any sst5 variant under normal-fed conditions (Fig. 5a). Similarly, food deprivation for 48 h did not alter HPT expression levels of sst5 variants in SST-KO as compared with wild-type control mice. Of note, however, whereas 48 h-fasting decreased PIT mRNA levels of all sst5 variants (full-length and truncated) in wild-type (control) animals, in SST-KO mice full-length sst5 expression was not reduced, while the mRNA levels for the two truncated variants did decrease in fasted SST-KO (Fig. 5a).

Analysis of sst5 variant expression in PIT and HPT mouse cell cultures

Finally, to address the potential direct regulatory actions of SST and CST, we examined their effects on the expression levels of sst5 variants in primary PIT cell cultures (Fig. 5b) and in the mouse HPT cell line N6 (Fig. 5c). Use of qrtRT–PCR indicated that sst5 and sst5TMD2 mRNAs were expressed in both PIT and HPT cell cultures (Fig. 5b, c, respectively), whereas mRNA for sst5TMD4 and sst5TMD1 were not detected in cultured cells, in line with the low levels of expression for these variants observed in whole PIT and HPT extract compared to those of sst5 and sst5TMD2 (Fig. 2d). Interestingly, results from these

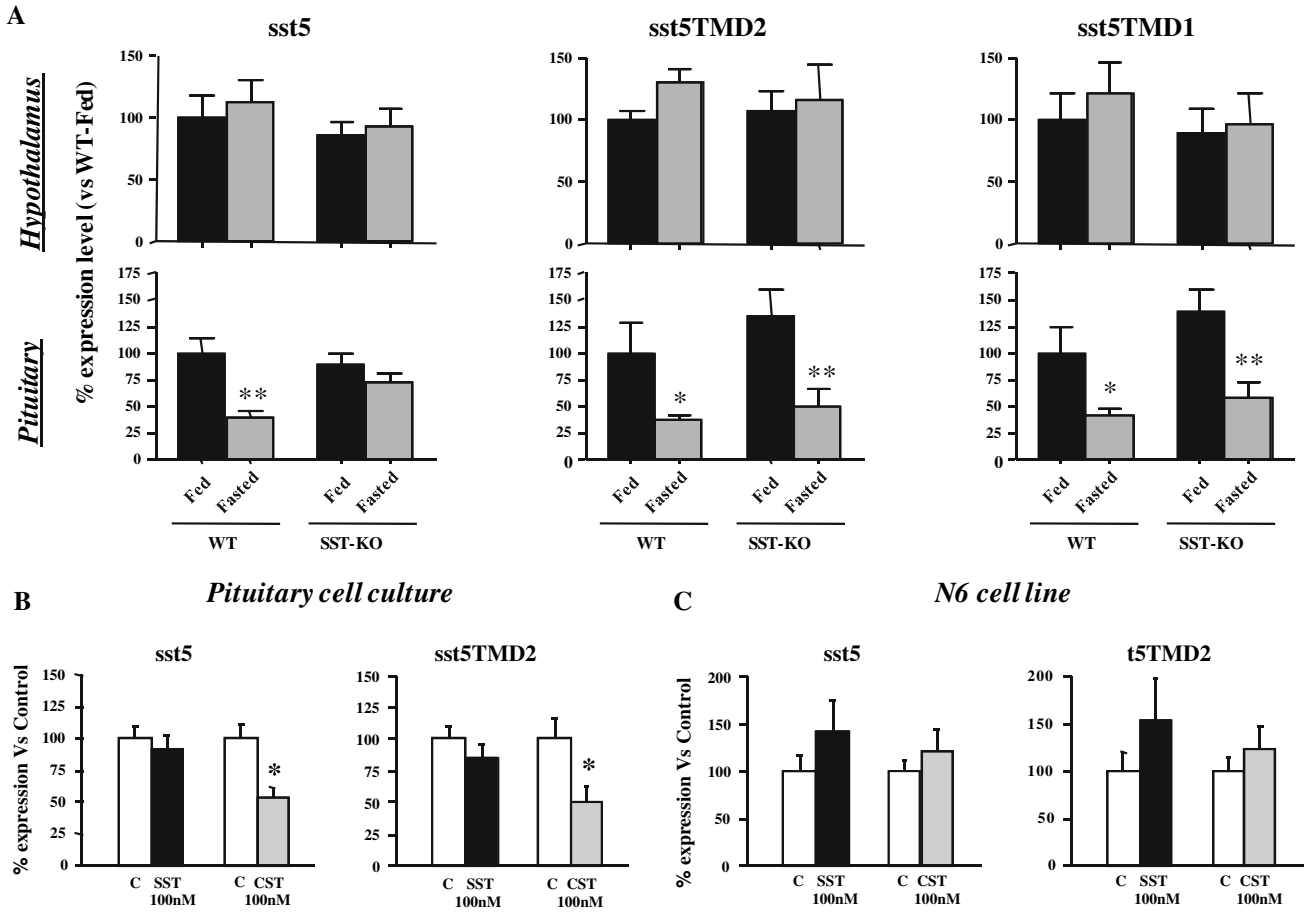

Fig. 5 Regulation of hypothalamic and pituitary mRNA levels of full-length and truncated mouse sst5 variants by their endogenous ligands, somatostatin (*SST*) and cortistatin (*CST*). **a** sst5, sst5TMD2 and sst5TMD1 mRNA levels in the hypothalamus (*top panel*) and pituitary (*bottom panel*) of fed and fasted (48 h) SST+/+ (*WT*) and SST−/− (SST-KO) male mice ($n = 5$–8 mice/group). **b**, **c** Effects of 24-h treatment of SST or CST (100 nM) on sst5 and sst5TMD2 mRNA levels in primary pituitary cell cultures from male mice (**b**) and in the murine hypothalamic cell line N6 (**c**). The mRNA copy number was determined by qrtRT–PCR, and the values were adjusted by cyclophilin A copy number as an internal control. Values are expressed as % of fed WT mice (**a** set at 100%) or as % of control-treated cells (**b**, **c** set at 100% within experiment). Values represent the mean ± SEM of $n = 5$–7 mice/group (**a**) or three independent experiments (**b**, **c** 3–4 replicates/treatment/experiment). *Asterisks* (*$P < 0.05$; **$P < 0.01$) indicate values that differ within genotype in response to fasting (**a**) or from control-treated cells (**b**, **c**)

treatments indicated a differential regulatory ability for SST and CST. Indeed, SST treatments did not significantly modify PIT or HPT mRNA levels of sst5 and sst5TMD2 variants in cultured cells, a situation similar to that found in whole PIT and HPT of wild-type controls compared with SST-KO mice (Fig. 5b, c). In contrast, CST treatments reduced by half the PIT mRNA levels of both sst5 and sst5TMD2 variants (Fig. 5b), while having no effect on HPT sst5 variants (Fig. 5c).

Discussion

The GPCRs superfamily of receptors, which includes the sst1-5 family, is associated with a number of important physio-pathological functions [41–43]. This explains the increasing interest in this research area, which is also supported by the fact that roughly 30% of marketed drugs target GPCRs, as is the case for synthetic SST analogs used to control cell growth and hormonal hypersecretion in pituitary adenomas and other neuroendocrine tumors [44, 45]. However, despite their biological importance and extensive study, there are still functions associated with these receptors that cannot be explained by the GPCRs known to date. Accordingly, over the last years several laboratories have been searching for novel, additional GPCR variants, and this has led in some cases to the identification of new, functional variants of GPCR bearing fewer than the typical seven TMDs that constitute the hallmark for this receptor superfamily [21, 22, 46, 47]. In particular, our laboratory has recently identified two new spliced variants of the human sst5 with unique properties

that could contribute to mediate SST and CST signaling in normal and tumoral cells [26]. Given that rodents are widely used to study the physiological importance of gene products, in the present study we followed the lead of our previous work on human sst5 and focused our efforts on the identification and characterization of new variants of sst5 in rats and mice. As a result, we have isolated, for the first time, novel variants of rodent sst5 with different size and sequence as compared to the full-length sst5s that are generated by splicing of cryptic introns at the sst5 mRNA. Specifically, we have identified three truncated sst5 variants in mouse (named sst5TMD4, sst5TMD2 and sst5TMD1) and one in rat (named sst5TMD1), displaying unique properties and the latter, mouse and rat sst5TMD1 showing high inter-specific nucleotide and amino acid sequence identity.

GPCRs are commonly encoded by genes with an exon-intron gene structure, wherein alternative splicing, especially within the C- and N-terminal tails, originates proteins with different lengths and sequences, although generally conserving the typical seven TMDs [48, 49]. In striking contrast, the new truncated sst5 variants described herein are not originated by classical alternative splicing, since the sst5 gene lacks canonical introns (starting with GT and ending with AG, the so-called GT-AG rule) within its CDS [50]. Thus, the generation of new truncated sst5 variants involves the presence of cryptic introns with non-canonical donor and acceptor splice sites resulting in new receptors that maintain the same N-terminal region as full-length sst5s, but bear different, shorter C-terminal tails with 4, 2 or 1 TMD (mouse sst5TMD4, sst5TMD2 or mouse and rat sst5TMD1, respectively) arising from a splicing of a portion of the canonical CDS and a portion of the 3′-UTR. These findings were not completely unexpected based on the fact that previous results from other laboratories studying different systems had suggested the existence of additional sst5 variants [12, 51]. Also, generation of gene products involving non-canonical cryptic introns is not rare, and over the last years several gene products derived from this type of splicing are being identified [52, 53], including the new truncated human sst5 variants reported by our group [26]. Interestingly, generation of this type of spliced proteins has been shown to involve new signals and mechanisms, which are still poorly understood and require further study [54, 55]. These include the presence of GC-rich motifs in the spliced genes, which could enhance the recognition of non-canonical introns resulting in new gene products [53, 56]. In fact, it has been reported that the relative gene density increases more than tenfold as GC content increases from 30 to 50% [56], as is the case for the sst5 gene in several species [66.3%, 64.8%, 64.4%, 58.16% and 55.07% for human, baboon, pig, rat and mouse, respectively (Genbank accession nos. NM_001053, EF639293, NM_001038229, NM_012882 and NM_011425.1, respectively)]. Indeed, while the overall GC content of the human and mouse genome is $\sim$40%, it is noteworthy that the GC content in the full-length and truncated sst5 variants spans from 55 to 60%, thus supporting the idea that the sst5 gene is a suitable target for the splicing machinery of non-canonical events.

An initial analysis of the expression pattern of the full-length and truncated sst5 variants in normal and tumoral tissues showed a wide but differential distribution of these receptors, which was tissue- and variant-dependent. Specifically, our results using qrtRT-PCR show that the overall predominant sst5 variants at the central and peripheral levels are full-length sst5 and sst5TMD2, which are highly expressed in endocrine/metabolic tissues as HPT, PIT, digestive tract, etc., whereas the other truncated receptors were less frequently and abundantly expressed. In fact, at the central level (HPT, cortex and cerebellum), we found that the expression of sst5 variants were also differentially distributed, with sst5 being the most abundant receptor in HPT and cerebellum, followed by sst5TMD2 and sst5TMD1, while mRNA levels of sst5TMD4 were not detected in those tissues. Interestingly, and similar to the results shown previously by others [57], sst5 mRNA levels were not detected in mouse cortex; however, quite interestingly, all truncated sst5 variants were present at different levels in this tissue (sst5TMD2 $\gg$ sst5TMD4 $>$ sst5TMD1), suggesting that truncated sst5 variants may play a physiologically important role in this tissue. Therefore, future studies need to be performed to elucidate the possible function of the truncated variants in these tissues.

Examination of the subcellular distribution of 3xHA-tagged receptors expressed in CHO-K1 cells by confocal microscopy indicated that all truncated sst5 variants display a predominant cytoplasmic localization, whereas full-length sst5 is mainly located at the plasma membrane, a situation that closely mimics that found recently for human sst5 variants [26]. Nonetheless, a proportion of all of the truncated sst5 variants were also located at the plasma membrane in an amount that seems sufficient to convey a potent ligand-induced response that is comparable to that exerted through the full-length receptor (see below). Although intracellular localization of GPCR is a common transient event due to the typical process of ligand-induced endocytosis or heterotypic interaction of receptors [58, 59], the question arises, why do truncated sst5 variants have a different subcellular localization than full-length sst5? A plausible explanation could be related with recent data showing that the signaling properties and cellular distribution of GPCR can be influenced by GPCR interacting proteins (GIP) that assemble with the intracellular regions

of the receptors, most notably with the C-terminal tail [18, 19, 60–62]. Although the C-terminal tails of GPCRs were promptly recognized as being important for the fine-tuning of G protein activation, their roles as 'magic' hooks to fish for GPCR-associated proteins have only recently been established. In this scenario, it seems reasonable to link this property with the observation that many GPCR splice variants [46, 63–65], including the human [26], mouse and rat sst5 variants (this study), differ in their C-terminal tail, and therefore, it could be speculated that the C-termini of these truncated sst5 variants could associate with distinct intracellular scaffolding/signaling molecules, which would selectively define their specific subcellular localization and signal transduction properties. Although additional studies are obviously required to validate this idea, our hypothesis is supported by recent functional and immunolocalization studies using AtT20 and HEK293 cells expressing the full-length sst5 or truncated C-terminal mutants of the receptor, indicating that the C-terminal tail motifs are crucial in the intracellular localization and trafficking regulation of the sst5 [61, 66] and also by a recent study showing that different regions of the sst5 are associated with the activation of different signaling pathways [67].

In spite of the fact that all novel sst5 variants possess less than seven TMDs and display different C-terminal tails than the full-length sst5, these truncated receptors maintain an intact N-terminus that has also been found to contribute to the ligand-induced activation mechanism. In fact, previous results of our laboratory have shown that human sst5 truncated variants exhibit a similar, but reduced ability to be activated by SST and CST compared with the full-length sst5 [26]. Therefore, to investigate whether the rodent sst5 variants were functional, we analyzed the cellular response ($[Ca^{2+}]_i$ and cAMP measurement) of CHO-K1 cells transfected with each of the sst5 variants after treatment with the endogenous ligands, SST and CST. Application of a Ca^{2+} imaging approach demonstrated that, despite their truncated nature, sst5TMD4/2/1 variants were functional receptors able to mediate increases in $[Ca^{2+}]_i$. Specifically, $[Ca^{2+}]_i$ levels were increased with SST and CST treatment in ~40–45% of the cells transfected with full-length sst5, which is consistent with previous reports showing a similar $[Ca^{2+}]_i$ increase in response to SST or a specific sst5 agonist in AtT-20 cells expressing sst5, as well as in cell lines transfected with human sst5 [68, 69]. Interestingly, only a minor percentage of cells transfected with sst5TMD1 (~11–20%) responded to SST and CST showing similar profiles. In clear contrast, whereas sst5TMD4 was almost exclusively activated by SST and not by CST (48% vs. 2%), cells transfected with sst5TMD2 responded quite selectively to CST and not to SST (51% vs. 13%). These results closely resemble those found for human truncated sst5TMD5 and sst5TMD4, which, despite not being orthologues for the mouse receptors, displayed a strong functional analogy in that they were selectively activated by SST and CST, respectively [26]. This suggests that shortening of sst5 unveils molecular features that enable the selectivity of the responses to these strikingly similar ligands, although this seems to include species-specific features, which will require additional detailed analysis.

To further investigate the functionality of the truncated sst5 variants, we studied the levels of sst5-mediated cAMP accumulation after SST and CST challenge. Our results showed that SST but not CST significantly inhibited forskolin-induced cAMP production in cells transfected with sst5TMD2 and 1. In clear contrast, SST tended to potentiate forskolin-induced cAMP accumulation in cells transfected with full-length sst5 and sst5TMD4. Although this latter observation did not reach statistical significance, this trend is in line with previous data showing that mouse and human sst5 could cause a stimulatory effect on cAMP accumulation in response to SST or specific sst5 agonists in some [68, 70, 71], but not all [70] cellular systems. All together, our results clearly demonstrate that truncated sst5 variants are functional, as shown by their ability to mediate selective, ligand-induced regulation in $[Ca^{2+}]i$ and cAMP levels in transfected cells, and that the different structure/sequence of each variant could result in the distinct functional properties shown by each individual receptor. Nevertheless, although these functional results are not conclusive, they provide primary, convincing evidence for the relevant ability of the new truncated sst5 receptors with 4, 2 and 1 TMD to mediate selective activation of intracellular signaling pathways in response to specific ligands (SST vs. CST). In fact, these results are consistent with previous findings showing that comparable splice variants with less than seven TMDs, such as the human truncated sst5 variants sst5TMD5 and sst5TMD4 [26], conserve the ability to convey selective ligand-induced responses. Furthermore, even receptor variants containing only the extracellular region of TMD receptors [i.e., corticotrophin-releasing factor receptor type 2 α (CRFR2α)] [23] have been reported to be capable of binding and modulating ligand activity.

SST and CST mediate a variety of biological effects, but the most well known are those occurring at the HPT and PIT level (i.e., inhibition of GH, TSH and ACTH release) [2, 5, 43]. Our results using qrtRT-PCR show, for the first time in mice, that the expression levels (copy number/0.1 μg total RNA) of the full-length sst5 are the highest in the PIT and HPT compared with other tissues. A similar situation was found for the truncated sst5TMD2 and sst5TMD1 receptors, whereas the expression levels of the sst5TMD4 variant were extremely low at the HPT and PIT level. Subsequently, use of different mice models under

distinct metabolic conditions revealed that truncated sst5 variants are differentially regulated in the PIT and HPT depending of the metabolic insult considered (i.e., fasting and obesity). Specifically, we observed that the expression level of both sst5TMD2 and 1 variants were downregulated in the PIT after a 12 h-fasting period, whereas HPT mRNA levels were unresponsive to this metabolic insult similar to that previously observed with the full-length sst5 variant [29]. However, we cannot exclude the possibility that our measurement of whole hypothalamic extracts may have masked nuclei-specific changes (arcuate, paraventricular, ventromedial) in sst5 variant expression in the different mouse models analyzed in the present study. Of note, other studies have also reported similar changes in pituitary sst expression in the fasted male rat [72], suggesting that fasting-mediated changes in pituitary sst5 responsiveness (plausibly including truncated receptor variants) could be common across species and thus that this phenomenon is likely to be of functional relevance. Actually, in contrast to the parallel regulation of full-length and truncated sst5 variants in the PIT in response to fasting, obesity (DIO and ob/ob mice) did not alter expression levels of sst5 variants in the HPT and PIT, indicating that, depending on the metabolic insult (fasting vs. obesity), sst5 variants are also differentially regulated in a tissue-specific manner.

These results raised the question of which factors may contribute to the regulation of PIT and HPT sst5 variants expression in response to metabolic stress. One factor may be endogenous SST, because previous studies have indicated that fasting and obesity could be associated with changes in SST tone [2, 32, 73–75], and SST can directly regulate the expression of its own receptors [4, 76, 77]. Therefore, we sought to determine if loss of endogenous SST (SST-KO compared with SST-intact control mice) could modulate the expression of truncated sst5 variants in PIT and HPT. We found that, similar to that seen with full-length sst5, mRNA levels of truncated sst5TMD2 and 1 variants were not altered in HPT and PIT of SST-KO mice under fed conditions compared with SST controls. These observations are also consistent with the in vitro results showing that SST did not alter mRNA levels of full-length sst5 or truncated sst5TMD2 in mouse primary PIT cell cultures and HPT N6 cells. In contrast, CST significantly decreased both full-length sst5 and truncated sst5TMD2 in PIT cell cultures, demonstrating, for the first time, that the modulation of full-length and truncated sst5 variants synthesis is ligand (SST vs. CST)- and tissue (PIT vs. HPT)-dependent in mouse. On the other hand, 48 h-fasting resulted in a significant decrease of truncated sst5TMD2 and 1 mRNA levels in the PIT of both SST-intact and SST-KO mice, whereas fasting inhibited full-length sst5 mRNA levels only in the PIT of SST-intact but not in SST-KO mice [29]. Therefore, our results demonstrate that the fasting-induced suppression of sst5 variants is variant-specific and that, at the PIT level, endogenous SST tone is a critically required regulator for the fasting-induced fall observed in the full-length sst5 synthesis, but not for the truncated sst5 variants.

In summary, we report for the first time the existence of truncated variants of the rodent sst5, one in rat and three in mouse, which may be of pathophysiological relevance because they: (1) are widely and distinctly expressed across tissues and they also display differential subcellular distribution compared to the full-length sst5; (2) are functional as shown by their ability to mediate ligand-selective changes in $[Ca^{2+}]_i$ and cAMP production; (3) their expression is regulated in a tissue-specific manner (central vs. systemic) by changes in hormonal and metabolic environment (i.e., fasting and lack of endogenous SST); (4) their expression is also regulated in a ligand-dependent manner, since CST but not SST was able to induce a downregulation on the sst5 variant mRNA levels in primary PIT cell cultures. The increasing multiplicity of protein networks associated with GPCRs generates more complexity in the understanding of the physiology, pharmacology and pathology related to this receptor family. However, the diversity of new functional sst may also shed some light into this complexity and could pave the way to discover novel drugs and tools to treat some endocrine pathologies associated with the SST- and CST-axis such as pituitary and other neuroendocrine tumors, cancer, immune disorders, etc.

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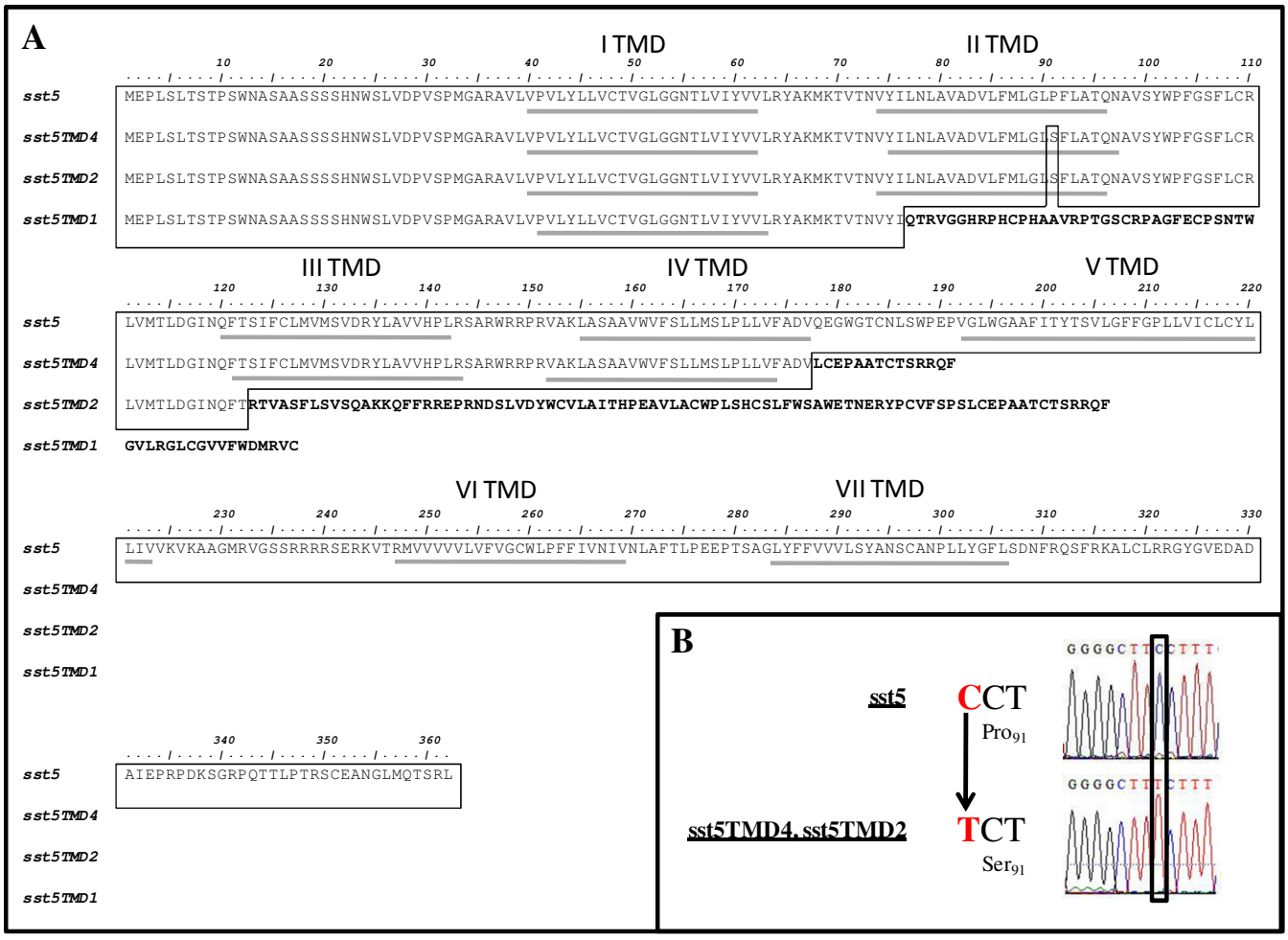
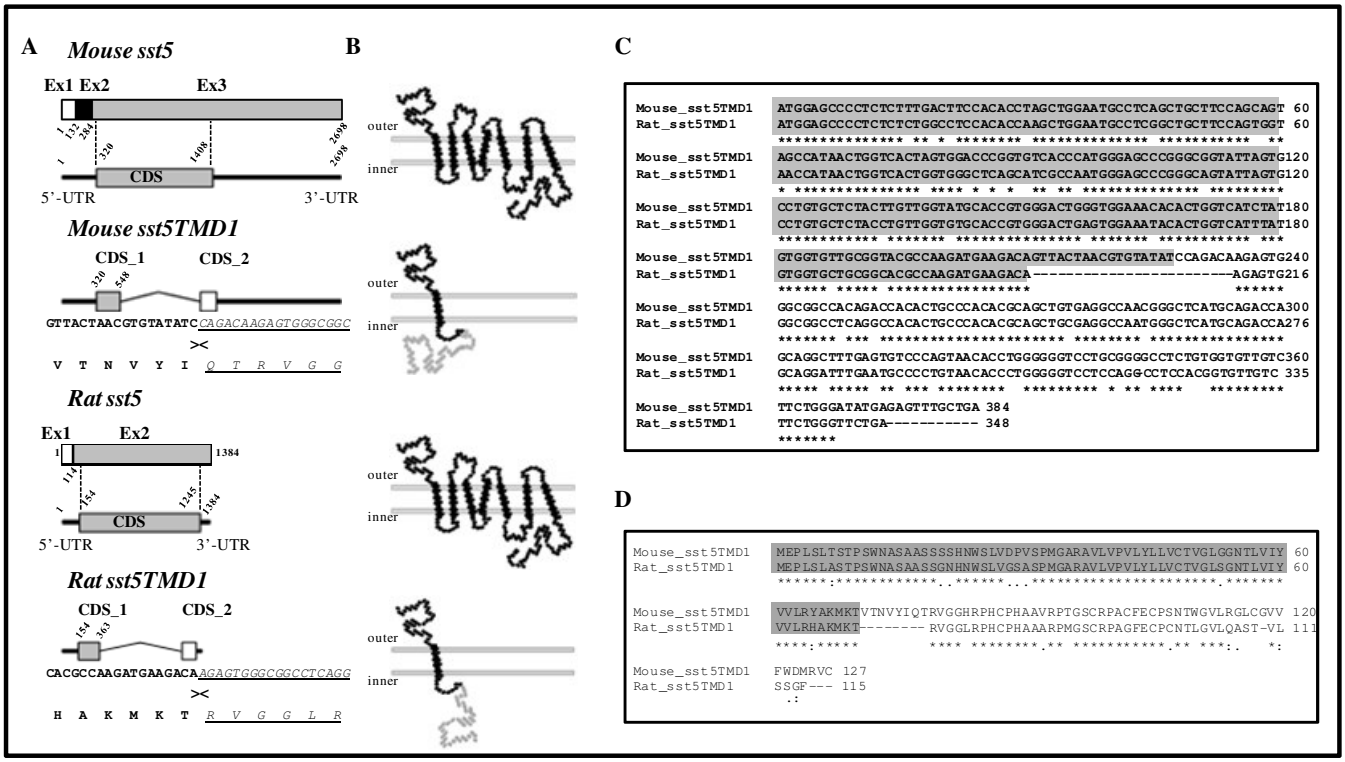
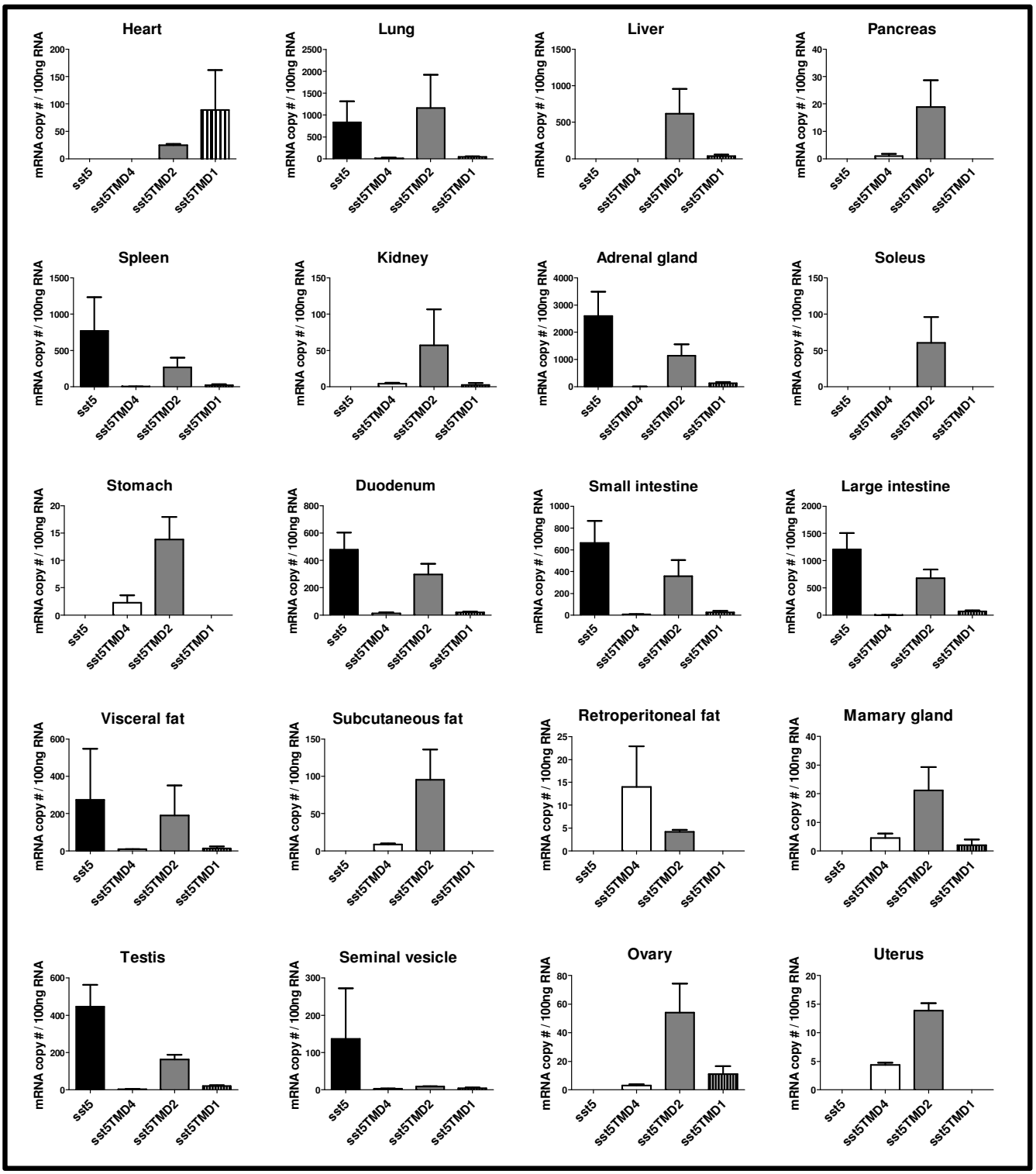
Supplemental Figure 1. A) Aminoacid sequence alignment of the mouse sst5 variants performed with Bioedit program (Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98). Black boxes delimit the aminoacid sequence that is identical across all sst5 variants. Bold letter outside the black boxes indicate the unique C-terminal tail of each sst5 variant. Predicted transmembrane domains (TMD) of each sst5 variant are indicated by grey lines. **B)** It should be noted that we consistently observed a single aminoacid change (Proline-91 in the full-length sst5 to Serine-91 in the truncated sst5TMD4 and sst5TMD2) produced by a single nucleotide change (C to U; CCT [Pro] to TCT [Ser]) which could be generated by a RNA editing process (for review: Blanc, V. and Davidson, N.O., (2003) C-to-U RNA Editing: Mechanisms Leading to Genetic Diversity. J Biol Chem. 278 (3) 1395-1398)**.**



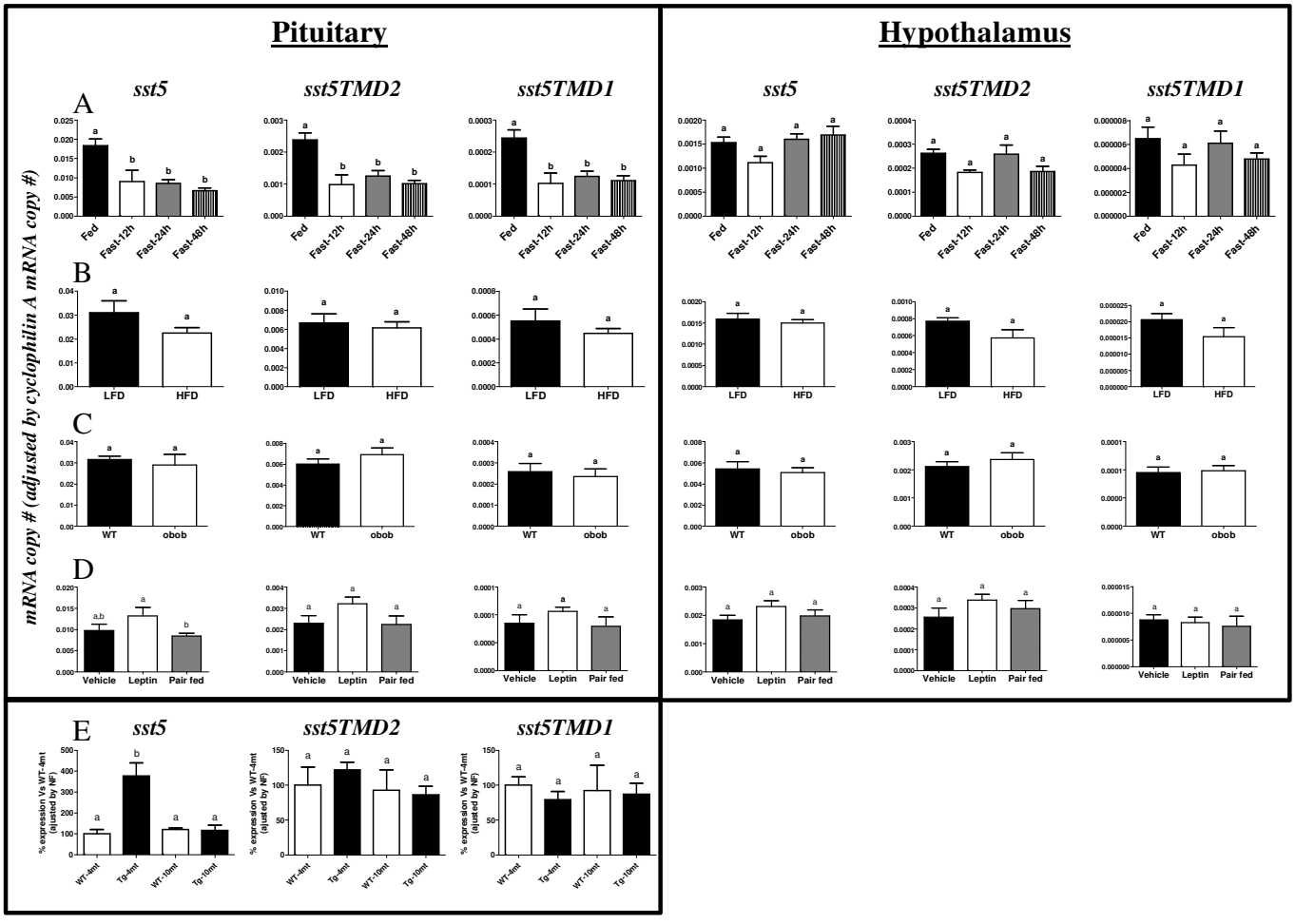
Supplemental Figure 2. **Molecular identification and characterization of the novel truncated rat sst5TMD1 variant and comparison with the mouse sst5TMD1 variant. A)** Schematic representation of the mouse and rat full-length sst5 and truncated sst5TMD1 mRNA variants. Mouse full-length sst5 is constituted by three exons (exon 1 [Ex1; white box; nucleotides # 1-132], exon 2 [Ex2; black box: nucleotides # 133-284] and exon 3 [Ex3; grey box; nucleotides # 285-2698]; coding region [CDS] located and codify within the Ex3 [nucleotides # 320-1408]) while rat full-length sst5 is constituted by two exons (exon 1 [Ex1; white box; nucleotides # 1-114] and exon 2 [Ex2; grey box: nucleotides # 115-1384]; CDS located and codify within the Ex2 [nucleotides # 154-1245]). Both, mouse and rat, sst5TMD1 are constituted by the same 5'UTR sequence (straight black line on the left) than full-length mouse or rat sst5, two coding regions (CDS_1 and CDS_2) and a 3'UTR sequence (straight black line on the right). The N-terminal regions of the CDS_1 (grey boxes) are similar between sst5 variants within species (full-length and sst5TMD1) but have different length (nucleotides #: 320-548 for mouse sst5TMD1 and 154-363 for rat sst5TMD1) while CDS_2 of each truncated sst5TMD1 variants (white boxes) are constituted by unique sequences originated by splicing of a portion of the CDS and/or 3'-UTR of the full-length mouse or rat sst5. The nucleotide and amino-acid sequence of the junction between the CDS_1 and CDS_2 of the truncated sst5TMD1 variants are shown below the corresponding schematic representation of each sst5 variant. **B)** Predicted transmembrane domain structure of full-length and truncated sst5TMD1 variants in mouse and rat. In grey are shown the differential C-terminal tails of the truncated sst5TMD1 variants compared with full-length sst5 variants. **C)** Nucleotide sequence alignment of the mouse and rat sst5TMD1 variants performed with ClustalW (www.ebi.ac.uk/clustalw/). Grey highlight represents the identical nucleotide sequence of the CDS_1 of the mouse and rat sst5TMD1 variants. Non-highlighted sequences represent the CDS_2 of each variant. The high interspecific homology (88% in nucleotide sequence) between the mouse and rat sst5TMD1 variants is indicated by asterisks. D) Aminoacid sequence alignment of the mouse and rat sst5TMD1 variants performed with ClustalW. Grey highlight represents the identical aminoacid sequence of the CDS_1 of the mouse and rat sst5TMD1 variants. Non-highlighted sequences represent the aminoacid sequence codify for CDS_2 of each variant. The high interspecific homology (81% in aminoacid sequence) between the mouse and rat sst5TMD1 variants is indicated by asterisks. Single point represents a conserved substitution in aminoacid sequence. Double point represents a semi-conserved substitution in aminoacid sequence.



Supplemental Figure 3: **Absolute cDNA copy number/0.1µg total RNA of full-length and truncated sst5 variants transcripts in systemic tissues**: heart, lung, liver, pancreas, spleen, kidney, adrenal gland, soleus, stomach, duodenum, small intestine, large intestine, fat (visceral, subcutaneous and retroperitoneal), mammary gland, testis, seminal vesicle, ovary and uterus of C57Bl/6J mice, as determined by quantitative real time RT-PCR (qrtRT-PCR). Values represent means ± SEM (n=5 mice).



Supplemental Figure 4: Regulation of hypothalamic (right panel) and pituitary (left panel) mRNA levels of full-length and truncated sst5 variants in various mice models under different metabolic conditions. A) Time course for the effect of fasting on the expression levels of sst5 variants. The animals were subjected to food deprivation (fast) for different time intervals: 12, 24 and 48h. Mice fed *ad libitum* served as controls (fed). B) Effect of diet-induced obesity (DIO) on the expression levels of sst5 variants. DIO mice were fed a high fat diet (HFD) for 16 weeks to induce overweight; mice fed a low fat diet (LFD) served as controls. C) Effect of lack of leptin (ob/ob mice) on the expression levels of sst5 variants. Lean littermates wild-type (WT) mice were used as controls. D) Effect of leptin infusion (7-days) on the expression levels of sst5 variants of adult ob/ob mice. Vehicle-infused ob/ob animals served as controls. In addition, because leptin treatment evoked a significant reduction in daily food intake, a group of ob/ob mice infused with vehicle and pair-fed along with leptin-treated animals was used as (additional) control. E) sst5 variants mRNA levels in Transgenic (Tg) MT-GHRH mice at 4 (hyperplastic pituitary) and >10 (adenomatous pituitary) months of age, and age-matched WT controls. Expression levels of sst5 variants were obtained by quantitative real time RT-PCR (qrtRT-PCR). Values are expressed as Mean ± SEM of mRNA copy number adjusted by Cyclophilin A mRNA copy number (used as internal control; n=5-10 mice/group; A-D) or adjusted by the Normalization factor (NF) obtained using GeNorm software (E, reference 34 of the present manuscript). Groups with different *superscript letter* are statistically different.



Article II

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Somatostatin and its receptors contribute in a tissue-specific manner to the sex-dependent metabolic (fed/fasting) control of growth hormone axis in mice

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Córdoba-Chacón J, Gahete MD, Castaño JP, Kineman RD, Luque RM. Somatostatin and its receptors contribute in a tissue-specific manner to the sex-dependent metabolic (fed/fasting) control of growth hormone axis in mice. *Am J Physiol Endocrinol Metab* 300: E000–E000, 2011. First published October 13, 2010; doi:10.1152/ajpendo.00514.2010.—Somatostatin (SST) inhibits growth hormone (GH) secretion and regulates multiple processes by signaling through its receptors sst1–5. Differential expression of SST/ssts may contribute to sex-specific GH pattern and fasting-induced GH rise. To further delineate the tissue-specific roles of SST and sst1–5 in these processes, their expression patterns were evaluated in hypothalamus, pituitary, and stomach of male and female mice under fed/fasted conditions in the presence (wild type) or absence (SST-knockout) of endogenous SST. Under fed conditions, hypothalamic/stomach SST/ssts expression did not differ between sexes, whereas male pituitary expressed more SST and sst2A/2B/3/5A/5TMD2/5TMD1 and less sst1, and male pituitary cell cultures were more responsive to SST inhibitory actions on GH release compared with females. This suggests that local pituitary SST/ssts can contribute to the sexually dimorphic pattern of GH release. Fasting (48 h) reduced stomach sst2A/B and hypothalamic SST/sst2A expression in both sexes, whereas it caused a generalized downregulation of pituitary sst subtypes in male and of sst2A only in females. Thus, fasting can reduce SST sensitivity across tissues and SST input to the pituitary, thereby contributing jointly to enhance GH release. In SST-knockout mice, lack of SST differentially altered sst subtype expression levels in both sexes, supporting an important role for SST in sex-dependent control of GH axis. Evaluation of SST, IGF-I, and glucocorticoid effects on hypothalamic and pituitary cell cultures revealed that these hormones could directly account for alterations in sst2/5 expression in the physiological states examined. Taken together, these results indicate that changes in SST output and sensitivity can contribute critically to precisely define, in a tissue-dependent manner, the sex-specific metabolic regulation of the GH axis.

somatostatin receptors; fasting; somatostatin-knockout mice; pituitary; hypothalamus; stomach

SOMATOSTATIN (SST) is a cyclic tetradecaneuropeptide that was initially isolated from hypothalamus (HPT) based on its capacity to inhibit growth hormone (GH) secretion from pituitary (PIT) somatotropes (6). SST has also been shown to be expressed in peripheral tissues, where the gastrointestinal tract (GIT) is the primary source of circulating SST (13). SST exerts pleiotropic effects, including modulation of neurotransmission, metabolism, and immune function, as well as inhibition of endocrine and exocrine secretion (13, 34, 49). SST exerts these effects through a family of five G protein-coupled receptor subtypes with seven transmembrane domains (TMD), termed sst1–5, which are encoded by separate genes (39, 49, 54). In addition, functional splice variants of ssts have been identified with seven TMD (39, 43, 65) or fewer, including the truncated sst5 variants recently identified in humans by our group (sst5TMD5 and sst5TMD4) (16) and in mice (sst5TMD4, sst5TMD2, and sst5TMD1) (12). Like SST, sst subtypes are expressed throughout the body, including the brain, PIT, and GIT, where the relative abundance of sst subtypes as well as SST is dependent on the species, tissue, sex, and nutritional state analyzed (34, 39, 49).

Experimental studies in humans, rodents, and other mammals have demonstrated that GH is released in a pulsatile manner, which is clearly different between sexes (19, 23, 40, 66). Specifically, under normal (fed) conditions, GH release in males tends to be highly organized, with high amplitude pulses and low baseline values. In contrast, GH release from females is more disorganized, with increased pulse frequency and elevated baseline values. Once secreted into the bloodstream, it is also clear that the sexually dimorphic pattern of GH exerts sex-specific effects on structural growth as well as liver function (68). Based on data generated mainly from rats, it has been hypothesized that one of the mechanisms responsible for elevated baseline GH release in females could be a reduction in SST output from its main tissue sources (HPT and GIT) (1, 10, 34, 40, 41, 44, 50, 62). This is supported by the observation that SST-knockout male mice display a feminized pattern of ultradian GH rhythm with an increase in interpulse GH release (28), which is associated with a more feminine pattern of hepatic gene expression (38). GH is also elevated in male and female SST-knockout mice, and in females, PIT expression of the main stimulatory receptors involved in GH secretion [GH-releasing hormone receptor (GHRH-R) and ghrelin receptor (GHS-R)] (30) is elevated. Interestingly, enhanced GH output in the absence of endogenous SST led to an increase in IGF-I only in females (30). Although SST is clearly important in maintaining the sexually dimorphic pattern of GH release and action, the relative contribution of tissue-specific SST/ssts expression remains to be clarified.

Changes in SST/ssts may also be involved in the fasting-induced rise in GH. However, the effect of fasting on circulating GH levels has been reported to be species dependent. Specifically, whereas fasting suppresses GH pulse release in

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male rats (34, 64), it enhances GH release in the majority of mammalian species studied to date, including humans and mice (22, 36, 34, 35). It has been suggested that the fasting-induced rise in GH could be due in part to downregulation of SST/sst signaling (34–36, 47, 52, 59, 60, 63), since a reduction in hypothalamic SST and pituitary and hypothalamic sst subtype expression (mainly sst2 and sst5) have been observed in rats and mice (34–36). The apparent role that SST plays in the sexually dimorphic pattern of GH release, coupled with data suggesting that changes in SST/sst expression contribute to the fasting-induced GH release, led us to conduct the current study with the aim of further delineating the tissue-specific roles of SST/sst in these processes by evaluating the expression pattern of SST and sst subtypes and variants (sst1, -2A, -2B, -3, -4, -5, -5TMD4, -5TMD2, and -5TMD1) in the HPT, PIT, and GIT of male and female mice under fed and fasted conditions in the presence or absence of endogenous SST [wild type, ($SST^{+/+}$) vs. SST-knockout ($SST^{-/-}$)]. In addition, a mouse HPT cell line, N6 (3), and primary PIT cell cultures from male and female mice were used to ascertain whether SST can regulate the expression of its own receptors in this specific context.

MATERIALS AND METHODS

In vivo animal models. All experimental procedures were approved by the Animal Care and Use Committees of the University of Cordoba and University of Illinois at Chicago. Male and female C57Bl/6J mice were purchased from Charles River Laboratories (Barcelona, Spain) or Jackson Laboratory (Bar Harbor, ME) at 8 wk of age. SST-knockout mice in a C57Bl/6 background were generously provided by Dr. Ute Hochgeschwender (Oklahoma Medical Research Foundation, Oklahoma City, OK) (71), and these mice were bred to C57Bl/6J mice to establish an experimental colony, as reported previously (30, 31). Mice were housed under standard conditions of light (12:12-h light-dark cycle) and temperature (22–24°C), with free access to tap water and food (standard rodent chow; LabDiet, St. Louis, MO). Mice were handled daily at least 1 wk prior to euthanasia to acclimate them to personnel and handling procedures and were euthanized by decapitation, without anesthesia, under fed conditions unless otherwise specified. At 9–11 wk of age, male and female mice were weighed, and food was withdrawn (0800–0900) from a subset of mice, whereas the remaining mice received food ad libitum ($n = 4$–10 mice/genotype/treatment group). Forty-eight hours later, mice were weighed and killed by decapitation without anesthesia. Blood and tissues (stomachs, PIT and HPT) were immediately collected, processed, frozen in liquid nitrogen and stored at −80°C for further analysis, as described below.

Assessment of circulating hormones. Trunk blood was immediately mixed with MiniProtease inhibitor (Roche, Nutley, NJ) and placed on ice and centrifuged, and plasma was stored at −80°C until analysis of GH (mouse/rat ELISA; Diagnostic System Laboratories, Webster, TX), IGF-I (IGF-I 100T RIA kit; Nichols Institute Diagnostic, San Clemente, CA), and corticosterone (Immunodiagnostic Systems, Fountain Hills, AZ) levels.

In vitro cell models. To investigate whether expression of PIT and HPT sst subtypes could be directly regulated by SST, IGF-I, and glucocorticoids, we used primary PIT cell cultures and the HPT N6 cell line (3). Briefly, PITs of adult male and female C57Bl/6J mice (10 wk of age) were dispersed into single cells and cultured in serum containing α-medium (Invitrogen, Barcelona, Spain), as described previously (12, 29, 31). N6 cells were cultured in monolayer in serum containing α-medium, as described previously (32). After 24 h of culture (200,000 cells/well, 24-well plates, maintained at 37°C in an atmosphere of 5% CO_2), medium was removed and cells were preincubated in serum-free medium for 1–2 h, and subsequently the medium was replaced with serum-free medium alone (controls) or containing SST-14 (100 nM), IGF-I (10 nM), or dexamethasone (a synthetic glucocorticoid, 10 nM) for an additional 24 h (3–5 wells/treatment/experiment). Then, total RNA was extracted and reverse-transcribed for determination of mRNA levels by quantitative real-time (qrt)RT-PCR (see below).

RNA isolation, reverse transcription, and gene expression analysis by qrtRT-PCR. Tissues and pituitary cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) with deoxyribonuclease treatment. The amount of RNA recovered was determined using the Ribogreen RNA Quantification Kit (Molecular Probes, Eugene, OR). Total RNA (1 μg for whole tissues and 0.15 μg for pituitary cell cultures) was reverse-transcribed using random hexamer primers with enzyme and buffers supplied in the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNA was treated with ribonuclease H, and duplicate aliquots (1 μl) were amplified by qrtRT-PCR, where samples were run against synthetic standards to estimate mRNA copy number. Details regarding the development, validation, and application of a qrtRT-PCR to measure expression levels of mouse transcripts have been reported previously (12, 29, 30). Briefly, thermocycling and fluorescence detection were performed using a Stratagene Mx3000p real-time PCR machine. The final volume of the PCR reaction was 25 μl, including 50 ng of sample, 12.5 μl of brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA), 1 μl of each primer (10 μM stock solution), 0.375 μl of the reference dye, and 10.375 μl of dH_2O. Thermal cycling profile consisted of a preincubation step at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 30 s), annealing (61°C, 1 min), and extension (72°C, 30 s). Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. To determine the starting copy number of cDNA, RT samples were PCR amplified, and the signal was compared with that of standard curve run on the same plate. Standard curves consisted of 1, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies of synthetic cDNA template for each of the transcripts of interest. Standard curves were generated by the Stratagene Mx3000p Software, and the slope of a standard curve for each template examined was between −3.31 and −3.35 (r^2 of the standard curve between 0.997 and 1.002), indicating that the efficiency of amplification was close to 100%, meaning that all templates in each cycle were copied. Specific sets of primers used in this study to measure expression levels of GH, SST, and all sst isoforms/variants (sst1, sst2A, sst2B, sst3, sst4, sst5, sst5TMD4, sst5TMD2, and sst5TMD1) are shown in Supplemental Table S1 (Supplemental Material for this article is available at the *AJP-Endocrinology and Metabolism* web site). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (used as housekeeping gene), where cyclophilin A mRNA levels did not significantly vary between experimental groups, within tissue type (data not shown).

Statistical analysis. Raw data were evaluated for heterogeneity of variance, and where found, values were log-transformed. Samples from all groups within an experiment were processed at the same time; therefore, the in vivo effects of sex/genotype/fasting and the in vitro effects of SST, IGF-I, and dexamethasone were assessed by one- or two-way ANOVA followed by a Newman-Keuls test for multiple comparisons or by Student's t-test, as appropriate. $P < 0.05$ was considered significant. All data are expressed as means ± SE. The in vivo effects of sex/genotype/fasting were obtained from a minimum of four animals per group. Results from in vitro studies were obtained from at least three separate independent experiments carried out on different days and with different cell preparation. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Silver Spring, MD).

RESULTS AND DISCUSSION

Relationship between tissue-specific expression of SST and sst subtypes on pituitary GH synthesis and release in male and female mice. As reviewed previously, one of the factors involved in the sexually dimorphic pattern of GH release could be SST/ssts system (1, 5, 10, 19, 23, 34, 40, 41, 44, 50, 61, 62, 66). To explore the tissue-specific contribution of this system in the sexually dimorphic pattern of GH release, the expression levels of HPT and stomach SST and sst subtypes were compared between male and female mice under fed conditions. The absolute mRNA levels for each transcript are shown in Table 1. As reported previously (29, 51), SST is highly expressed in the stomach and HPT of mice (Table 1), whereas the relative expression of the sst subtypes varied between tissues. Specifically, in the HPT, sst2A > sst4 ≥ sst1 > sst3 > sst5 ≥ sst2B > sst5TMD1 = sst5TMD2 (mRNA levels of sst5TMD4 were not detected; Table 1), whereas in stomach, sst2A > sst2B > sst4 > sst3 > sst1 = sst5 (mRNA levels of all truncated sst5 variants were not detected; Table 1). Although expression patterns/levels varied broadly between HPT and stomach, there were no differences in the expression level of each transcript between sexes (Fig. 1), a finding that lessens the potential role of SST/sst in these tissues in the sexually dimorphic pattern of GH release. Nevertheless, the possibility that our measurement of whole hypothalamic and stomach extracts may have masked changes in SST/sst expression in specific HPT nuclei (periventricular, paraventricular arcuate) (34, 37, 67) or GIT regions and cell types (13) should not be excluded. In fact, cell-specific regulation of SST expression is supported by the observation of some (11, 20, 28, 41, 42) but not all authors (5, 26) showing that female rats and mice have lower immunodetectable SST in specific hypothalamic regions (i.e., median eminence, periventricular nucleus) compared with males, where these changes are associated with a reduction in SST mRNA levels in the periventricular nucleus but not in the arcuate nucleus. Also, Zhang et al. (72) observed that sst1 mRNA-expressing cells were two- to threefold greater in the arcuate, but not in the ventromedial, nucleus of the HPT in males compared with female rats. Thus, future (neuro)anatomic studies in these animal models may help to unequivocally ascertain this apparent lack of sex-related differences in SST/ssts at the HPT and stomach levels.

However, in addition to SST produced in the HPT and stomach, our laboratory and others have shown that SST mRNA is present in the PIT (14, 28, 30, 51), suggesting that local production of SST could also play a relevant role in controling somatotrope function. In fact, our present results show that PITs of females express less SST compared with male mice (496 ± 132 copies in males vs. 140 ± 43 copies/0.05 μg total RNA in females, $P = 0.026$; Table 1 and Fig. 1), which is consistent with a previous report showing similar results using nonquantitative RT-PCR methods (28). The relative expression levels of the PIT sst subtypes differed from that observed in the HPT and stomach, with sst2A ≥ sst5 ≥ sst3 = sst1 = sst4 > sst5TMD2 > sst2B > sst5TMD1 (mRNA levels of sst5TMD4 were not detected; Table 1), in both males and females. In this case, comparison of the levels of expression for each transcript between sexes revealed a marked divergence, since female PIT expressed lower levels of mRNA for the primary sst subtypes mediating the actions of SST on GH release, sst2A, and sst5 (34) as well as other sst isoforms/variants (sst2B, sst3, sst5TMD2, and sst5TMD1; Fig. 1). These results are consistent with reports on rats (24, 72) showing that the female PIT expresses less sst2 and sst5 compared with male PIT. Although the majority of sst subtypes were lower in the female PIT, the expression of sst1 was greater compared with males, as reported previously by others (55) but not observed by all (72). It remains to be determined what factors are responsible for these sex-dependent differences in SST/sst expression in the PIT. The limited data available demonstrate that direct application of estrogen to female rat PIT cell cultures increases sst2A/B and sst3 mRNA levels, whereas it decreases sst1 expression (15). It should be noted that these changes are opposite to that observed between males and females in mice (present study) and rats (24, 72), suggesting that either estrogen is not directly responsible for these differences or the timing or dose of estrogen used did not appropriately mimic the actions of estrogens in vivo. There is also a possibility that nonsteroidal gonadal factors may play a role in these differences, as shown previously for sex-dependent difference in hypothalamic expression of SST and GHRH in rats (27).

In the current study, the overall reduction in the expression of most sst subtypes, where sst2 and sst5 copy numbers alone

Table 1. *Absolute cDNA copy number/0.05 μg total RNA of SST and all sst transcripts in different tissues (hypothalamus, pituitary, and stomach) of male and female mice as determined by quantitative real-time RT-PCR*

	Hypothalamus		Pituitary		Stomach	
	Male	Female	Male	Female	Male	Female
SST	133,150 ± 20259	113,784 ± 14,472	496 ± 132	140 ± 43*	55,687 ± 7,551	39,896 ± 5,513
sst1	1,965 ± 283	2,146 ± 313	1,025 ± 102	1,831 ± 306*	5 ± 0	6 ± 0
sst2A	12,931 ± 2156	12,097 ± 1,525	5,906 ± 1,250	2,782 ± 620*	16,407 ± 289	14,160 ± 2,128
sst2B	334 ± 34	347 ± 38	249 ± 39	116 ± 23*	1,010 ± 139	559 ± 92
sst3	1,090 ± 107	950 ± 99	2,422 ± 234	1,384 ± 239*	26 ± 3	30 ± 7
sst4	5,392 ± 645	3,529 ± 695	1,195 ± 103	802 ± 133	116 ± 14	130 ± 21
sst5	508 ± 47	539 ± 64	3,300 ± 508	1,732 ± 305*	4 ± 1	4 ± 1
sst5TMD4	ND	ND	ND	ND	ND	NM
sst5TMD2	37 ± 1	38 ± 2	860 ± 58	548 ± 41*	NM	NM
sst5TMD1	45 ± 7	51 ± 9	40 ± 2	27 ± 1*	NM	NM

Values represent means ± SE of the mRNA copy number of each transcript (n = 4-9 mice/sex/tissue). SST, somatostatin; sst, SST receptor isoform/variant; ND, not detected; NM, not measured. *$P < 0.05$, values that differ within sex.

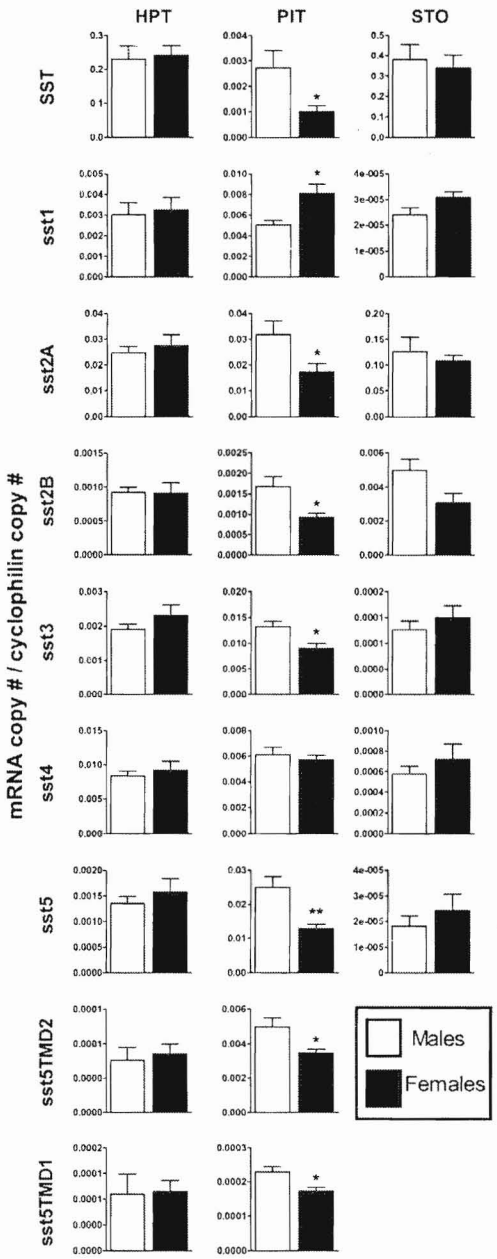

Fig. 1. Somatostatin (SST) and SST receptor isoform/variant (sst) expression in hypothalamus (HPT), pituitary (PIT), and stomach (STO) of fed male (open bars) and female (filled bars) wild-type C57Bl6/J mice as assessed by quantitative real-time (qrt)RT-PCR. Values represent means ± SE (n = 4–9 mice/sex/tissue) of absolute mRNA copy numbers (adjusted by cyclophilin mRNA copy number). Symbols ($^*P < 0.05$, $^{**}P < 0.01$) indicate values that differ within sex.

account for ~50% of total sst transcripts (Table 1), supports the hypothesis that the female PIT would be far less responsive to the inhibitory actions of SST. However, it could be argued that these differences are due to the differential contribution of the various PIT cell types in female vs. male PIT (4). To circumvent this problem, we tested the impact of SST on GH synthesis and release from primary PIT cultures prepared from female and male mice using a single dose of SST (100 nM). This dose has previously been demonstrated to exert a maximal inhibitory effect on GH synthesis and/or release in other species [i.e., rat, pigs, primate and human (34, 48, 56)]. Specifically, we found that female mice were indeed significantly less sensitive to the inhibitory actions of SST compared with males (Fig. 2), a response similar to that observed in primary PIT cultures prepared from rats (44, 53). However, future studies using different doses of SST would need to be performed to unequivocally ascertain whether the observed differences between sexes are related to a change in sensitivity (ED_{50}) and/or in the number of receptors (B_{max}).

To our knowledge, this is the first report simultaneously comparing by absolute quantitative methods (qrtRT-PCR) the expression levels (copy number) of SST, as well as all sst isoforms/variants, between male and female mice in key target tissues [central (HPT) and peripheral (PIT and stomach)] for SST actions. The fact that, under normal-fed conditions, *1*) no changes in HPT/stomach SST or sst subtype levels were observed between fed male and female mice, *2*) male PIT expressed more SST and sst2A/2B/3/5A/5TMD2/5TMD1 and less sst1 compared with PIT of females, and *3*) in vitro somatotropes from male mice are more sensitive to the inhibitory actions of SST on GH synthesis and release, compared with female somatotropes, suggests that local PIT alterations of the SST/sst may directly and relevantly contribute to the well-known sexually dimorphic pattern of GH release observed in most mammalian species. However, it should be noted that we do not know whether the changes observed in SST/sst subtype mRNA levels are translated into changes in functional protein levels. This would require species-specific and highly sensitive antibodies for each of the mouse sst subtypes, where such reagents are currently unavailable.

Role of SST and sst subtypes in the fasting-induced rise in circulating GH levels in males and females. Fasting has been shown to suppress HPT SST mRNA in male mice and ewes (21, 29, 46), consistent with the current observations showing an overall reduction in HPT SST mRNA, which reached significance in females (Fig. 3). In addition, fasting suppressed HPT sst2A expression in males and females, whereas the

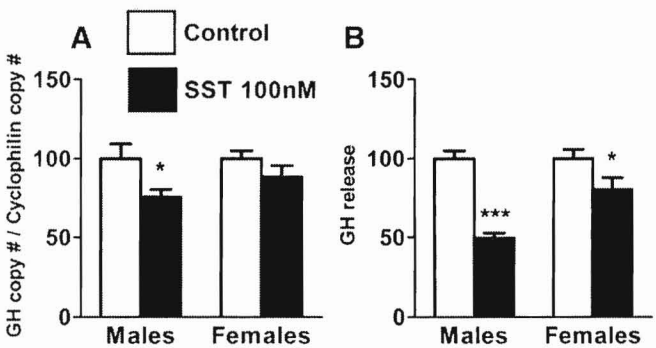

Fig. 2. Effect of 24-h treatment of SST (100 nM; filled bars) on growth hormone (GH) expression (*A*) and release (*B*) in primary PIT cell cultures from male and female mice. GH mRNA copy numbers were determined by qrtRT-PCR, and the values were adjusted by cyclophilin A copy number as an internal control, whereas GH release levels were determined by commercial ELISA. Values represent the mean ± SE of 3 independent experiments (3–5 wells/treatment/experiment) and are expressed as percentage of vehicle-treated controls (set at 100%; open bars) within experiment. Symbols ($^*P < 0.05$, $^{***}P < 0.001$) indicate values that differ from vehicle-treated controls.

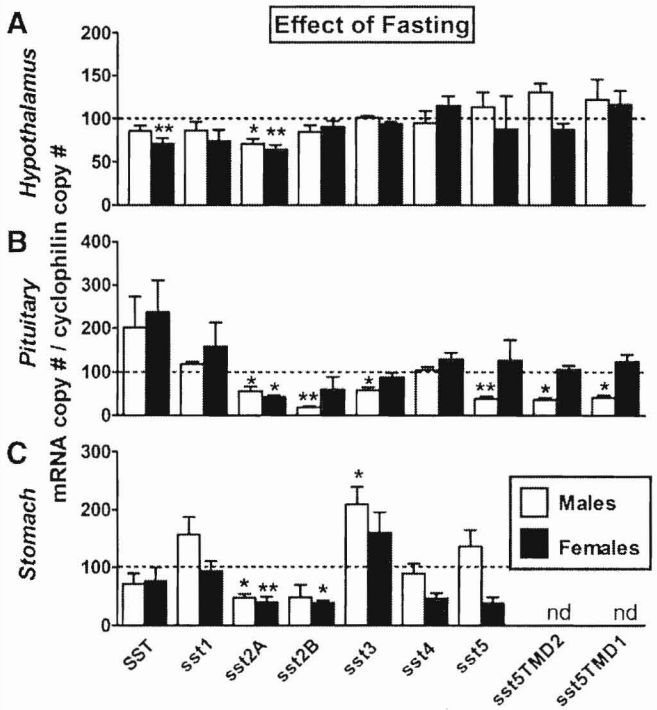

Fig. 3. Effect of fasting on SST and sst in HPT (*top*), PIT (*middle*), and STO (*bottom*) of male (open bars) and female (filled bars) mice. mRNA copy numbers were assessed by qrtRT-PCR. Values are shown as relative percentage of male or female fed control mice (shown by the dotted line set at 100%) and represent the mean ± SE of 4–9 mice/sex/tissue. Symbols ($*P < 0.05$, $**P < 0.01$) indicate values that significantly differ from fed controls within sex.

expression levels of the other sst subtypes were not significantly altered (Fig. 3*A*). Since sst2A is the major sst isoform within the HPT (Table 1), these results indicate that the rise in GH levels observed in fasting is associated not only with a reduction in SST expression but also with a downregulation of its central signaling. Evidence supporting an inhibitory role of sst2A signaling in GH-negative feedback has been provided by a study showing that MK-0677 (a GHS-R agonist) stimulated c-fos expression in HPT, and this response could be inhibited by GH pretreatment in intact but not in sst2A-null mice (55). Furthermore, since SST directly stimulated sst2A expression in a HPT cell line (N6; see Fig. 6*A*), it is not unreasonable to suggest that the specific changes in sst2A expression in response to fasting observed herein could be related, at least in part, to a reduction in local production of SST. In addition, the fall in circulating IGF-I and/or the rise in circulating glucocorticoids that occurs with fasting (33) may contribute to the downregulation of sst2A expression observed in fasting in that IGF-I increased whereas dexamethasone decreased sst2A expression but did not modify sst2B mRNA levels in mouse HPT N6 cell cultures (Supplemental Fig. S1*A*).

Fasting did not significantly alter stomach SST expression in male or female mice (Fig. 3*C*), which is consistent with a previous observation showing that SST mRNA levels were not changed in fasted rats (73). However, fasting downregulated the expression levels of the dominant sst subtypes sst2A and sst2B in both sexes, whereas sst3 mRNA levels were significantly increased only in females. These changes in stomach sst subtype expression may be indirectly involved in the rise in GH observed with fasting by promoting the production of the GH-releasing peptide ghrelin, which is produced primarily by the stomach (9, 25). This hypothesis is supported by *1*) the observation that SST and cortistatin (a peptide sharing high structural and functional similarities to SST), as well as octreotide (a sst2A preferring agonist), can downregulate circulating ghrelin levels in fed and fasted humans (2, 7) and rats (57) and *2*) our data showing that circulating ghrelin levels are elevated in male (29) and female (data not shown) SST-knockout mice.

At the level of the PIT, fasting did not significantly alter SST expression in male or female mice (Fig. 3*B*). However, fasting decreased PIT sst2A/2B/3/5/5TMD2/5TMD1 expression in males, whereas only sst2A mRNA levels were suppressed in females (Fig. 3*B*), a differential response that may be related to the already reduced PIT expression of sst subtypes in females compared with males (Fig. 1). Nonetheless, given that sst2A is the predominant sst subtype in the PIT and is clearly linked to the inhibitory effect of SST on GH release (34), downregulation of PIT sst2A, coupled with reduced HPT SST input, could represent a primary mechanism by which GH levels increase in response to fasting. The fact that fasting-induced downregulation in PIT sst2/sst5 expression has been observed in male rats (8, 34, 47) and nutrient deprivation has been shown to decrease the GH response to exogenous SST administration in rats, dogs, and humans (33, 52, 59, 60, 63) suggests that the fasting-induced changes in PIT responsiveness to SST is preserved across species. The mechanism by which these changes occur may be directly related to the well-characterized reduction in circulating IGF-I and rise in glucocorticoids observed with fasting (Ref. 36 and Fig. 4, *left*) since IGF-I can directly

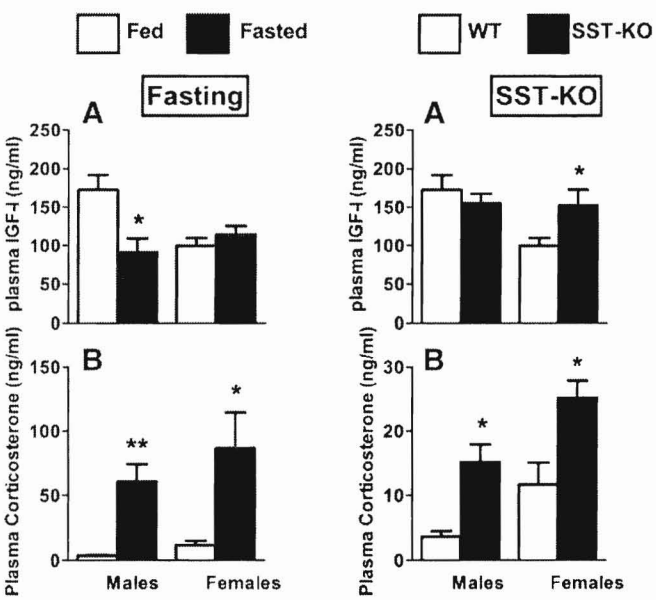

Fig. 4. Circulating IGF-I and corticosterone levels of fed (open bars) or fasted (filled bars) (*A* and *B*, respectively, *left*) and SST-intact (open bars) and SST-knockout (SST-KO; filled bars) (*A* and *B*, respectively, *right*) male and female mice. IGF-I and corticosterone values are represented as means ± SE (ng/ml; $n = 4–10$ mice/treatment-genotype/sex) and were determined by commercial RIA or ELISA kits, respectively. Symbols ($*P < 0.05$, $**P < 0.01$) indicate differences between fed and fasted mice or between SST-intact and SST-KO mice within sex.

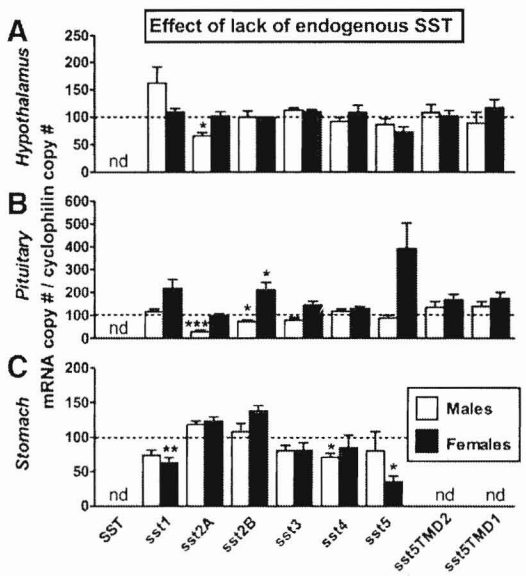

Fig. 5. SST and sst expression in HPT (*A*), PIT (*B*), and STO (*C*) of male and female wild-type (SST-intact control mice shown by the dotted line set at 100%) compared with male (open bars) and female (filled bars) SST-KO mice, as assessed by qrtRT-PCR. Values represent the mean ± SE of 4–9 mice/sex/tissue. Symbols ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) indicate values that significantly differ from SST-intact within sex. nd, Not detected.

upregulate the expression of sst2A (Supplemental Fig. S1*B*) as well as sst1, sst2B, and sst5 (36) in primary PIT cell cultures from male mice, whereas glucocorticoids directly inhibit sst2A expression (Supplemental Fig. S1*B*). The role of glucocorticoids is further supported by reports showing that glucocorticoids also decrease PIT sst2 mRNA levels in rats (45, 70) and the presence of consensus glucocorticoid response elements in the promoter of the mouse sst2 gene [as reviewed by Park et al. (45)]. Nevertheless, the actual contribution of systemic IGF-I input in modulating PIT sensitivity to SST in fasted female mice is lessened by the observation that fasting did not result in a significant reduction in total IGF-I levels in females (Fig. 4*A*, *left*), although we should not discard the possibility that changes in free (available) IGF-I levels may have occurred, based on studies in humans showing that short-term fasting is characterized by a decrease in bioavailable "free" IGF-I attributed in part to a rise in circulating IGF-binding protein-1 (17).

Role of endogenous SST in regulating its own receptor expression in male and female mice: relationship with circulating GH levels. Use of SST-knockout mouse models has enabled our laboratory and others to show that SST is required to suppress GH release in males and females and that these actions are critical to preserve the sex-dependent pattern of GH release and its subsequent actions (28, 30). Furthermore, we also discovered that loss of endogenous SST (SST-knockout) is more critical in female than in male mice in suppressing PIT expression of GH, GHRH-R, and GHS-R, resulting in a significant increase in circulating IGF-I levels and a reduction in HPT expression of GHRH, the latter likely due to enhanced negative feedback by GH/IGF-I (19, 36, 40). These sex-dependent differences in the response to SST loss may be related to the fact that in males, but not females, HPT cortistatin is increased, which could in part compensate for SST loss (30). In addition, an upregulation in PIT ghrelin expression as well as GHS-R expression found in female SST-knockout (Supplemental Fig. S2) may also contribute to the enhanced sensitivity of the female SST-knockout GH axis to SST loss, since we have reported recently that locally produced PIT ghrelin could directly participate in the regulation of PIT function independent of circulating ghrelin levels produced by the stomach, thereby acting as a positive ultrashort feedback loop to enhance or facilitate GH release (18). Perhaps some of these or other similar mechanisms are in place in humans in that GH release is more dramatic in women, compared with men, after arginine-mediated SST repression alone or combined with a ghrelin agonist (58, 69).

In the current study, we have extended these observations by examining the impact of SST-knockout on tissue-specific expression of sst subtypes in male and female mice. In general, the lack of SST had little impact on HPT sst subtype mRNA levels (Fig. 5), where only the expression of sst2A was significantly downregulated in SST-knockout males. Lack of SST may be directly responsible for the decrease in sst2A expression because, as mentioned above, SST increases sst2A mRNA levels in HPT N6 cells (Fig. 6*A*); but this alone is unlikely to fully account for the sex differences observed. In fact, using the same in vitro model system, we found that dexamethasone inhibits whereas IGF-I stimulates sst2A mRNA (Supplemental Fig. S1*A*). Since corticosterone levels are elevated in both male and female SST-knockout mice, although IGF-I is enhanced only in females (Fig. 4), we might speculate that the male-

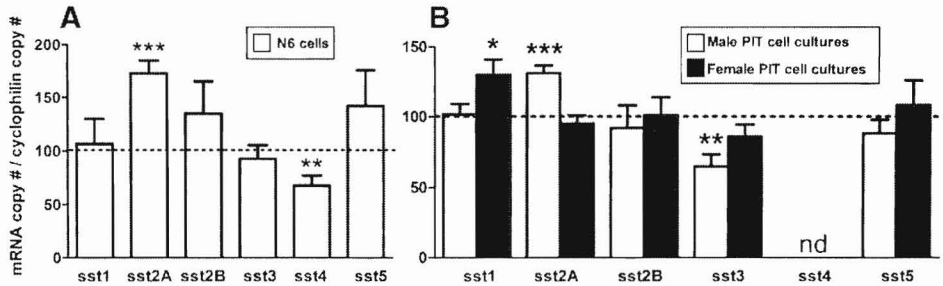

Fig. 6. Effect of 24-h treatment of SST (100 nM) on sst subtype expression in mouse hypothalamic N6 cells (*A*) and in primary PIT cell cultures (*B*) from male (open bars) and female (filled bars) mice. mRNA copy numbers were determined by qrtRT-PCR, and the values were adjusted by cyclophilin A copy number as an internal control. Values represent the mean ± SE of 3–4 independent experiments (3–5 wells/treatment/experiment) and are expressed as percentage of vehicle-treated controls [shown by the dotted line set at 100% in N6 cells (*A*) and in male and female mice (*B*)] within experiment. Symbols ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) indicate values that differ from the corresponding vehicle-treated controls.

specific reduction in PIT sst2A expression could in part be due to the direct inhibitory effect of glucocorticoids unopposed by changes in IGF-I.

In contrast to the HPT, lack of endogenous SST was associated with a decrease in stomach sst4 in males and a decrease in sst1 and sst5 mRNA levels in females (Fig. 5*C*). Although the expression levels of sst1, sst4, and sst5 in the stomach are low compared with sst2A (Table 1), we cannot discount the possibility that fewer sst subtypes would minimize the inhibitory impact of cortistatin (which is expressed in the GIT and is intact in SST-knockout mice) on ghrelin release (7).

At the level of the PIT, we observed that sst2A and sst2B were downregulated in males, whereas sst1, sst2B, and sst5 were upregulated in female SST-knockout mice. These results, taken together with previous reports (28, 30), reinforce the notion that SST is an important player in the sex-dependent differences in GH axis function (34). Downregulation of sst2 isoforms in male PIT may be directly mediated by loss of SST, reduction in IGF-I, and elevation of glucocorticoids, similar to that discussed for HPT sst2 regulation, in that sst2A expression is stimulated by SST and IGF-I (with sst2B expression also being stimulated by IGF-I) and suppressed by dexamethasone in primary PIT cell cultures (Supplemental Fig. S1*B*). We might speculate that the lack of change in sst2A expression and elevation of sst1, sst2B, and sst5 in PIT of female SST-knockout mice may be related to elevations in IGF-I, since our laboratory has shown that IGF-I can increase these receptor isoforms in mouse primary PIT cell cultures (36).

It should be mentioned that the tissue-specific and relative changes in the expression levels of sst subtypes in SST-knockout mice originally developed in the laboratory of Dr. Ute Hochgeschwender (71) are partially different from those generated using SST-knockout mice developed in the laboratory of Dr. Malcolm Low (28), as reviewed in detail elsewhere (34). It is possible that such differences are due to different strategies for gene deletion, background strain, time of day tested, age, light cycle, diet, stress, and/or analytical techniques used. Therefore, the assessment of the effect of somatostatin replacement in both somatostatin-knockout mouse models should be undertaken in future studies to unequivocally elucidate the precise contribution of endogenous somatostatin on the observed changes in the regulation of sst subtypes and GH axis function in both animal models.

Summary. The present results strongly support the hypothesis that the sexually dimorphic pattern of GH release is influenced largely by SST/ssts system, where the PIT appears to be a key component in this process, since female PITs express lower levels of SST and ssts and are relatively unresponsive to the inhibitory action of in vitro SST treatment compared with males. Our data also substantiate and extend the work of others implicating a reduction in SST tone in the fasting-induced rise in GH, which may involve a reduction in HPT SST expression as well as an overall downregulation in ssts within the HPT, PIT, and stomach; however, the specific changes in ssts were tissue and sex dependent. Studies examining the direct effects of SST, IGF-I, and dexamethasone on expression of these sst subtypes in a HPT cell line and in primary PIT cell cultures, coupled with the sex-dependent impact of fasting on these end points, may explain in part the sex-dependent impact of fasting on HPT and PIT sst expression. Finally, our data suggest that the GH axis of female mice is more sensitive to the loss of endogenous SST, particularly at the level of the PIT, showing upregulation of GH-stimulatory receptors and a general downregulation of ssts, likely leading to an increase in IGF-I. These changes were not observed in male SST-knockout perhaps because of a compensatory rise in HPT cortistatin. When viewed as a whole, these observations suggest that changes in SST output, as well as sensitivity, at multiple levels of the GH axis play a role in sex difference and metabolic regulation of GH release.

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DISCLOSURES

The authors have nothing to disclose.

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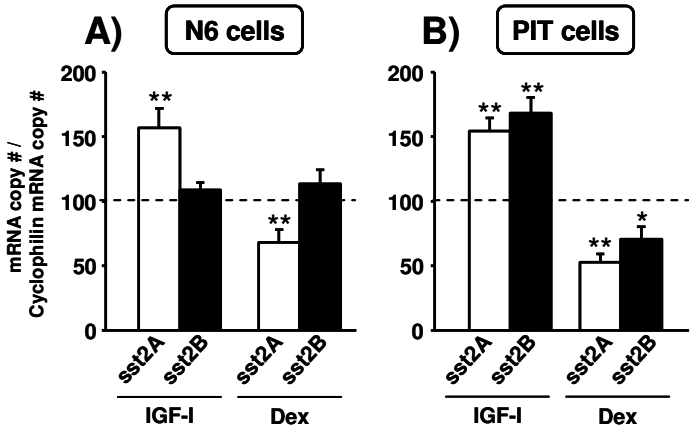
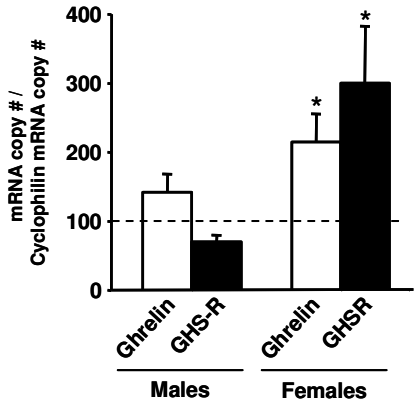
Supplemental Table 1: Mouse-specific primers for somatostatin (SST), all somatostatin receptor subtypes, growth hormone (GH), ghrelin, ghrelin receptor (GHS-R) and cyclophilin-A used for quantitative real-time, RT-PCR.

Template	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
SST	NM_009215.1	**Sense:** TCTGCATCGTCCTGGCTTT **Antisense:** CTTGGCCAGTTCCTGTTTCC	**Sn** 138 **As** 250	113
sst1	NM_009216	**Sense:** TGCCCTTTCTGGTCACTTCC **Antisense:** AGCGGTCCACACTAAGCACA	**Sn** 757 **As** 891	135
sst2A	NM_001042606	**Sense:** CCCATCCTGTACGCCTTCTT **Antisense:** GTCTCATTCAGCCGGGATTT	**Sn** 925 **As** 1058	134
sst2B	NM_009217.2	**Sense:** TGATCCTCACCTATGCCAACA **Antisense:** CTGCCTTGACCAAGCAAAGA	**Sn** 893 **As** 997	105
sst3	NM_009218.3	**Sense:** GCCTTCTTCGGCCTCTACTT **Antisense:** GAATGCGACGTGATGGTCTT	**Sn** 1292 **As** 1430	139
sst4	NM_009219.3	**Sense:** AGGCTCGTGCTAATGGTGGT **Antisense:** GGATGAGGGACACATGGTTG	**Sn** 860 **As** 980	121
sst5	NM_011425.2	**Sense:** ACCCCCTGCTCTATGGCTTT **Antisense:** GCTCTATGGCATCTGCATCCT	**Sn** 1215 **As** 1319	105
sst5TMD4	GQ359775	**Sense:** GTCCACCCTCTCCGCTCA **Antisense:** GCAGGTTCGCAGAGGACATC	**Sn** 415 **As** 545	131
sst5TMD2	GQ359776	**Sense:** CAGTTCACCCGTACTGTGGCAT **Antisense:** CACAGCTTCAGGGTGGGTAA	**Sn** 358 **As** 489	132
sst5TMD1	GQ359777	**Sense:** AACGTGTATATCCAGACAAGAGTGG **Antisense:** TCCCAGAAGACAACACCACA	**Sn** 217 **As** 368	152
GH	NM_008117	**Sense:** CCTCAGCAGGATTTTCACCA **Antisense:** CTTGAGGATCTGCCCAACAC	**Sn** 412 **As** 553	142
Ghrelin	NM_021488.4	**Sense:** TCCAAGAAGCCACCAGCTAA **Antisense:** AACATCGAAGGGAGCATTGA	**Sn** 163 **As** 288	126
GHS-R	NM_177330.3	**Sense:** TCAGGGACCAGAACCACAAA **Antisense:** CCAGCAGAGGATGAAAGCAA	**Sn** 1002 **As** 1072	71
Cyclophilin A	NM_0008907.1	**Sense:** TGGTCTTTGGGAAGGTGAAAG **Antisense:** TGTCCACAGTCGGAAATGGT	**Sn** 421 **As** 529	109

Supplemental figure 1: Effect of 24-h treatment of IGF-I (10nM) and dexamethasone (Dex; 10nM) on sst2A (white columns) and sst2B (black columns) expression in mouse hypothalamic N6 cells (A) and in primary pituitary cell cultures from male mice (B). mRNA copy numbers were determined by qrtRT-PCR, and the values were adjusted by cyclophilin A copy number as an internal control. Values represent the mean ± SEM of 2-5 independent experiments (3-4 wells/treatment/experiment) and are expressed as percentage of vehicle-treated controls (shown by the dotted line set at 100%) within experiment. Asterisks (*, p<0,05; **, p<0,01) indicate values that differ from the corresponding vehicle-treated controls.



Supplemental figure 2: Ghrelin (white columns) and GHS-R (black columns) expression in pituitary of male and female wild-type (SST-intact controls mice shown by the dotted line set at 100%) compared to male and female SST-KO mice, as assessed by qrtRT-PCR. Values represent the mean ± SEM of 4-7 mice/gender/tissue. Asterisks (*, p<0,05) indicate values that significantly differ from SST-intact within gender.



Article III

Cortistatin is not a somatostatin analogue but stimulates prolactin release and its deficit causes plasma insulin decrease and male-selective glucose impairment: role of ghrelin.

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Abstract

Cortistatin (CST) and somatostatin (SST) evolve from a common ancestral gene and share remarkable structural, pharmacological, and functional homologies. Although CST was considered a mere natural SST analogue acting through their shared receptors (sst1-5), emerging evidence indicates that these peptides might in fact exert unique roles via selective receptors (e.g. CST, not SST, binds ghrelin receptor GHS-R1a). Here, the specific roles of CST were investigated using a mice knockout (KO) model and primary pituitary cell cultures. Specifically, we present the first thorough endocrine-metabolic characterization of male/female CST-KO mice at the hypothalamic, pituitary and systemic (pancreas-stomach-adrenal-liver) levels. Also, CST effects on hormone expression and secretion were evaluated in primary pituitary cell cultures from male/female mice and female primates (baboons). These unveiled unique, unpredicted regulatory actions of CST on the pituitary-metabolic axis, distinct to those of SST. Specifically, CST exerts an unexpected stimulatory role on prolactin secretion, which is mediated via GHS-R1a, and likely relates to the decreased success in first-litter pup care at weaning. In addition, CST played important inhibitory actions on ACTH and growth-hormone axes, which are elevated in cort-/- mice and are inhibited by CST in vitro. Furthermore, CST deficit uncovered a major, gender-dependent role of this peptide in the regulation of glucose-insulin homeostasis, as it caused an overall impairment of insulin-mediated glucose clearance in male cort-/- vs. littermate-cort+/+. The fact that these actions are not mimicked by SST and are strongly gender-dependent offers new grounds to investigate the hitherto underestimated physiological relevance of CST in the regulation of endocrine and metabolic process.

Introduction

Cortistatin (CST) is a neuropeptide that shares a remarkable structural, pharmacological, and functional homology with somatostatin (SST) with which it appears to descend from a common ancestral gene [1-4]. Indeed, although CST and SST are encoded by two separate genes [1-5], their expression leads to the synthesis of two similar pre-pro-hormones [1, 3]. Moreover, like prepro-SST, prepro-CST is enzymatically cleaved to generate two main mature products: CST-14 and CST-29 in rodents, and CST-17 and CST-29 in humans, which are analogous to the corresponding mature SST peptides, SST-14 and SST-28, respectively [6]. Furthermore, all SST/CST products share two unique structural features: a disulfide bridge (that maintains their typical cyclic structure), and the FWKT motif, both of which are crucial for these peptides to exert their biological effects [6, 7], and, in particular, to bind with comparable affinity to a family of five receptors, the so-called somatostatin receptors or sst1-sst5 [8, 9]. This may explain why both peptides, SST and CST, have been mostly described to influence similarly a number of relevant processes at multiple levels, from the central nervous system (CNS), to peripheral, endocrine and non-endocrine tissues, yet, some distinct actions have also been described for each peptide [10-13]. Thus, SST, originally identified by its ability to inhibit the secretion of growth hormone (GH) from pituitary somatotropes [14], is nowadays recognized as a multifarious, widespread peptide able to regulate (patho)physiologically relevant functions as distinct as locomotor activity, insulin release, glucose homeostasis, or gastrointestinal motility [1, 13, 15-18]. Furthermore, long-acting synthetic SST analogues, such as octreotide and lanreotide, are commonly used as antitumoral drugs to treat pituitary adenomas (i.e. GH-secreting tumors), and their clinical value in other pathologies (i.e. retinopathies, obesity, and diabetes) [19-21] and malignancies (i.e. breast and prostate cancers) [16, 19, 22] is under intense study. On the other hand, the precise functional roles of CST are still less well understood. The limited data available so far regarding its endocrine activities indicate that CST, like SST, also inhibits GH secretion and influences insulin release [10, 12, 23, 24]. However, to date, no studies have thoroughly investigated the precise actions and potential physiological relevance of CST at the pituitary level and its metabolic interface, despite evidence suggesting that SST and CST are more than two "endocrine siblings" [12].

In fact, there is increasing evidence that CST can exert unique functions, distinct to those of SST, although the

underlying mechanisms are still poorly understood. For example, at the central nervous system (CNS), SST and CST expression does not overlap in some neuronal populations, and, if co-expressed in the same neurons, they are differentially regulated [25]. This may be related to the fact that, as opposed to SST, CST induces slow-wave sleep and hypomotility [5]. Likewise, CST exerts unique immunomodulatory and antiinflammatory actions not shared by SST in various animal models [26, 27]. Also, tissue expression patterns and regulation of both peptides do not overlap in human tissues [10, 28-30]. These and other results have led to the concept that CST may exert its unique actions through specific receptors not effected comparably by SST, yet the nature of those receptors remains elusive [1, 31, 32]. As mentioned above, much of the functional versatility of SST, and likely of CST, is mostly related to their complex set of widely distributed sst receptors [8, 9]. ssts are often present simultaneously in the same cells, where they functionally interact with each other or with other G-protein coupled receptors (GPCR) forming homo- and/or hetero-dimers to activate different signaling cascades and mediate multiple actions [8, 9, 33-36]. Interestingly, some sst-subtypes are expressed in tissues where SST is not present, suggesting that CST may be the natural ligand for these receptors therein. Moreover, our group has recently identified novel truncated sst5 variants in human [37] and mice [38], which display differential ability to mediate CST- and SST-induced cellular responses (i.e. Ca2+ signaling). Other alternative, non-sst receptors have also been suggested as potential mediators for specific CST actions, such as the Mass-related MrgX2 receptor, which is nevertheless not present in rodents and is more likely to act as a proadrenomedulin receptor [39, 40]. In contrast, earlier work showing that CST, but not SST, is able to displace acylated ghrelin from its bindings sites in the pituitary (likely GHSR-1A) [41], suggested a unique functional interaction between CST and ghrelin systems, not mimicked by SST [31], a potential link that has been substantiated by studies on the antiinflammatory actions of CST and ghrelin [42].

In this scenario, it is important to note that available evidence indicates that CST substantially reproduces, but not fully mimics the “in vitro” effects of SST on pituitary secretions of human and animal models [10, 12], thus raising the unsolved question of whether CST is a mere natural endocrine analogue of SST, or whether it possesses its own, physiologically relevant regulatory actions on pituitary and other endocrine/metabolic secretions. In an attempt to answer this question, in the present study we used male and female cortistatin deficient (cort-/-) mice to develop the first thorough endocrine-metabolic characterization of these animals at the hypothalamic, pituitary and systemic (pancreas, stomach and liver) levels, as compared with their wildtype-littermate controls (cort+/+). In addition, primary pituitary cell cultures from male and female mice and female primates [olive baboons (Papio anubis)] were used to further ascertain whether CST can directly regulate the expression and secretion of pituitary hormones. Our results unveil that CST exerts unique, unpredicted regulatory actions on the pituitary-metabolic axis which are distinct to those previously established for SST.

Materials and methods

In vivo animal models

All experimental procedures were approved by the Animal Care and Use Committees of the University of Cordoba. Cortistatin-deficient (cort-/-) mice were created by targeted disruption of the cort gene in embryonic stem cells followed by the derivation of transgenic cort-/- mice as recently described [43-45]. Somatostatin-deficient (sst-/-) mice were generously provided by Dr. Ute Hochgeschwender [46]. C57Bl/6J male and female mice, heterozygous for the cort-null or sst-null mutations, were bred to C57Bl/6J mice purchased from Charles River (Barcelona, Spain) to generate cort+/+, cort+/- and cort-/- or sst+/+, sst+/- and sst-/- littermates mice for this study. Genotypes of the sst-colony were determined by PCR of tail-snip DNA, using the primers and genotyping protocol previously reported [47]. Mice from the cort-colony were PCR-genotyped at weaning using genomic DNA (35ng) of tail-snip and three primers (Supplemental table 1: primer 1: 5’–AGTGCACCTGCTCGGTTCGTCTC–3’, primer 2: –TCCCTGACAGACCCAGGCTAGGA–3’ and primer 3: 5’–CATGTCGCACGTGCATGTCGAC–3’; 400nM each, annealing temperature: 61ºC) in a 25ul-PCR reaction that could generate a product of 159bp corresponding to the wild type-allele(+) and/or a product of 300bp corresponding to the KO-allele(-) using the MJ Mini™ Gradient Thermal Cycler (BioRad, Madrid, Spain). All mice were housed under standard conditions of light (12-h light/dark cycle) and temperature (22–24C), with free access to tap water and food (standard rodent chow; SAFE-diets, Barcelona, Spain). Adult mice were handled daily at least 1 week prior to euthanasia to acclimate them to personnel and handling procedures and were sacrificed [at ~11 wk of age] by decapitation, without anesthesia, under fed conditions. Blood and tissues (cortex, hypothalamus, pituitary, stomach, and liver) were

immediately collected, processed, frozen in liquid nitrogen and stored at -80ºC for further analysis as described below.

Immunohistochemistry of pituitary cells

To examine the impact of CST deficiency on pituitary cell numbers (somatotropes, corticotropes lactotropes, gonadotropes and thyrotropes), whole pituitaries from a subset of male and female cort+/+ and cort-/- mice (n=4 mice/genotype) were fixed in 4% paraformaldehyde overnight, stored in 70% EtOH, paraffin embedded and sectioned (4µm) for immunocytochemistry. Specifically, pituitary sections were immunostained for GH, PRL, adrenocorticotropin (ACTH)/proopiomelanocortin-(ACTH precursor; POMC) and β-subunits of thyroid-stimulating (TSH) and luteinizing (LH) hormones using rabbit polyclonal antibodies from Dr. A.F. Parlow (NHPP,NIDDK) as previously described [48].

In vitro cell models (mouse and primate)

- *Experiment 1:* For assessment and comparison of basal GH, PRL and ACTH release, pituitaries of adult male and female cort+/+ and cort-/- mice [10-12 wk of age; n=4 (3-5 pituitaries pooled/experiment)] were dispersed into single cells and cultured in serum containing α-medium (Invitrogen, Barcelona, Spain), as previously described [38, 47, 49].

- *Experiment 2*: For studies examining the direct effects of CST on the expression of all mouse pituitary hormones [GH, PRL, POMC, β-subunits of LH, follicle-stimulating hormones (FSH) and TSH and the glycoprotein hormone α-subunit] and sst1-5 as well as on GH, PRL and ACTH release, pituitaries of normal adult male and female C57Bl/6J mice (10-12 wk of age; n= 3-5 pituitaries pooled/experiment, 4 independent experiments carried out in different days and with different cells preparation) were dispersed into single cells and cultured in serum containing α-medium (Invitrogen), as previously described [38, 47, 49]. In addition, pituitaries from randomly cycling female non-human primate [olive baboon (*Papio anubis*); 9-14 yr of age] were obtained after pentobarbital sodium overdose from control animals under Institutional Animal Care and Use Committee-approved studies conducted by other University of Illinois at Chicago investigators. Baboon pituitaries were enzymatically dispersed into single cells as previously described [47, 50, 51].

After 24h of culture (200,000 cells/well, 24-well plates, maintained at 37C in an atmosphere of 5% CO2), media was removed and cells pre-incubated in serum-free media for 1-2h and subsequently the media was replaced with serum-free media alone (experiment-1 and controls of experiment-2) or containing CST [CST-14 (in mouse) or CST-17 (in baboon); 100nM-dose previously identified to exert a maximal effect on pituitary hormone synthesis and/or release in other models [52, 53]] for an additional 24h (3-4 wells/treatment/experiment). Then, media was removed and frozen for subsequent analysis of mouse or human GH, PRL and ACTH levels and total cellular recovered for determination of mRNA levels, as described below. In addition, an antagonist for the GHS-R1a (BIM-28163) was used in order to determine whether CST-mediated PRL release was signaled via this receptor.

Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$)

To measure $[Ca^{2+}]_i$ in response to CST, single cells were plated at a density of 50,000 cells onto 25-mm photoetched grid coverslips (Bellco Biotechnology, Vineland, NJ, USA) and processed as previously described [38] using a Nikon (Eclipse TE2000-E; Nikon, Tokyo, Japan) microscope with attached back thinned-CCD cooled digital camera (ORCA II BT; Hamamatsu Photonics, Hamamatsu, Japan).

Assessment of mRNA levels

Total RNA was extracted from tissues (cortex, hypothalamus, pituitary, stomach, and liver) or primary pituitary cell cultures, reversed transcribed and amplified by quantitative real-time RT-PCR (qrtRT-PCR). Details regarding development, validation and application of a qrtRT-PCR to measure expression levels of mouse and baboon transcripts have been previously described [38, 47, 48, 50, 54, 55]. Specific primer sequences, Genbank accession numbers and product sizes are provided as supplemental information (Table S1). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number for the mouse transcript of interest was adjusted by a normalization factor (NF) calculated from the mRNA copy number of 2-4 separate housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine ribosyltransferase (HPRT), beta-actin and/or cyclophillin-A, depending of the tissue analyzed] using the GeNorm 3.3 visual basic application for Microsoft Excel [http://medgen.ugent.be/~jvdesomp/genorm [56]] while mRNA copy number of the mouse and baboon transcripts analyzed in the primary pituitary cell cultures were adjusted by the mRNA copy

number of cyclophilin-A, where mouse NF or mouse/baboon cyclophylin-A levels did not significantly vary between experimental groups, within tissue type (data not shown).

Analysis of fertility and pups care in cort-/- mice, compared to cort+/+ mice

We compared the time to conception (by inspection of plugs), days of gestation and litter size of female cort-/- and cort+/+ mice. In addition, we compared the percentage of cort-/- and cort+/+ female mice that raise a litter successfully until weaning (21days). Female mice were mated twice [at 9-10 weeks (first crossbred) and at 13-30 (second crossbred) weeks of age].

Assessment of circulating hormones and metabolites

Glucose levels were determined in fresh blood samples using the Glucocard G+ glucometer (Arkray; Amstelveen, The Netherlands). The remaining trunk blood was immediately mixed with MiniProtease inhibitor (Roche; Barcelona, Spain) and placed on ice, centrifuged and serum was stored at −80 ◦C until analysis of hormones. Hormones were assessed using commercial ELISA kits for mouse GH, insulin, leptin, total-ghrelin (desacylated+acylated), acylated-ghrelin (Millipore; Madrid, Spain); ACTH, SST, glucagon (Phoenix Europe GmbH; Karlsruhe, Germany); corticosterone, IGF-I (Immunodiagnostics Systems; Boldon, UK); prolactin (PRL; Calbiotech, Spring Valley, CA, USA) and human GH, PRL (DSL, Inc., Webster, TX); ACTH (ALPCO; Salem, NH)]. Mouse LH was measured by specific RIA using a double-antibody method and radioimmunoassay kits kindly supplied by Dr. F. Parlow and as previously described [57]. In order to prevent rapid des-acylation of ghrelin after the plasma collection, hydrochloric acid was added to an aliquot of plasma at a final concentration of 0.05N.

Analysis of glucose metabolism in cort-/- and sst-/- mice: glucose and insulin tolerance tests (GGT and ITT)

GTT (glucose 1g/kg, ip; overnight fast condition) and ITT [insulin 1U/kg, Actrapid (Novo-Nordisk; Bagsvaerd, Denmark); fed condition] were performed in cort+/+ and cort-/- as well as in sst+/+ and sst-/- mice at 16-17 weeks of age between 0700h-1000h. One week prior to GTTs and ITTs, mice were acclimated daily to handling and brief restraint. Ten minutes prior to blood sampling topical anesthetic cream (2.5%lidocaine: 2.5%prilocaine) was applied to the tail. Glucose levels were assessed by Glucocard G+ glucometer prior to injection (t0) and 15, 30, 60 and 120 minutes after injection of glucose or insulin. In addition, circulating insulin levels were compared between cort-/- or sst-/- vs. controls (cort+/+ or sst+/+, respectively) mice.

Statistical analysis

Raw data were evaluated for heterogeneity of variance and where found, values were log transformed. Samples from all groups within an experiment were processed at the same time. The in vivo and in vitro effect of genotype [male and female cort-/- or sst-/- vs. controls (cort+/+ or sst+/+, respectively) mice] and the in vitro effects of CST were assessed by Student's t-tests. Comparison of the response to GTT and ITT was assessed by two-way-ANOVA followed by a Bonferroni test for multiple comparisons. All data are expressed as means ± S.E.M. $P<0.05$ was considered significant. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD, USA).

Results

Characterization of cortistatin deficient mice

To verify the correct genotype of each of the animals employed in this study, PCR products were run in an agarose gel (2%) and stained with ethidium bromide, and were thereby confirmed to correspond to the intact-allele (+; 159bp amplified with primers 1 and 2) and/or the null-allele (-; 300bp amplified with primers 1 and 3), and thus to derive from cort+/+, cort+/- or cort-/- mice, as shown in supplemental figure-1A. Expression of endogenous CST was further assessed by qrtRT-PCR to ensure the genotype of each mouse (supplemental figure-1B). Specifically, CST mRNA was undetectable in cerebral cortex and hypothalamus of male and female cort-/- mice. However, hypothalamic CST mRNA levels in cort+/- compared to cort+/+ were 62.6 ± 13.7% and 29.9 ± 6.9% in males and females, respectively, which is consistent with a previous report [43] showing that CST peptide levels of cort+/- mice were half of cort-intact controls in cerebral cortex, thus indicating there is limited or no compensation by the remaining cortistatin allele. It should be noted that we did not found any significant difference between cort+/+ and cort+/- mice in all the endpoints analyzed in the present study [levels of circulating hormones and metabolites, transcript expression levels, etc (data not shown)] and therefore, only comparisons between cort+/+ and cort-/- are shown and discussed below.

Effect of genotype on somatotrope, lactotrope, corticotrope, gonadotrope and thyrotrope axes functions

Inasmuch as CST and SST similarly bind to all sst1–5, and apparently share many actions at different targets, we first sought to determine whether CST deficiency was associated with alterations in pituitary hormones axes, as occurs in SST-KO mice [46, 47, 49, 58].

- *Somatotropes* (Fig. 1A): Circulating GH levels were significantly higher in male and female cort-/- mice compared with cort+/+ mice. Interestingly, as compared with cort+/+, the overall elevation in plasma GH levels was higher in female (six-fold) than in male (four-fold) cort-/- mice, an observation which may be related to the fact that pituitary GH mRNA levels were up-regulated only in female cort-/-. As a consequence, while mean basal circulating GH levels were higher in male than in female cort+/+ mice, the enhanced GH release caused by CST deficit in female mice resulted in GH levels comparable to those observed in male cort-/- mice. Differences in GH output between genotypes were not associated with changes in pituitary size or in the relative abundance of somatotrope cells, as assessed by gross morphology and by the proportion of GH immunopositive cells (table-1), respectively. The elevated plasma GH levels found in cort-/- did not result in increased body weight (Fig. 1E and table-2) or significant changes in circulating IGF-I levels (Fig. 1A) or in its hepatic expression (supplemental table-2) . Moreover, cort-/- mice exhibited the normal sexual dimorphism in body length that becomes evident during puberty. Organ weight [fat depots (visceral and subcutaneous), liver and pancreas] (table-2) and food intake [male cort +/+ 3.88 ± 0.12 vs. cort-/- 4.11 ± 0.15 g/day; female cort +/+ 3.78 ± 0.11 vs. cort-/- 3.59 ± 0.11 g/day] were similar between male or female cort-/- and cort+/+ mice

- *Lactotropes* (Fig. 1B): Circulating PRL levels were markedly decreased in both male and female cort-/- compared with cort+/+ mice. Plasma PRL levels were higher in female cort+/+ compared with male cort+/+ mice, and this gender-related divergence was maintained in cort-/- mice. Differences in

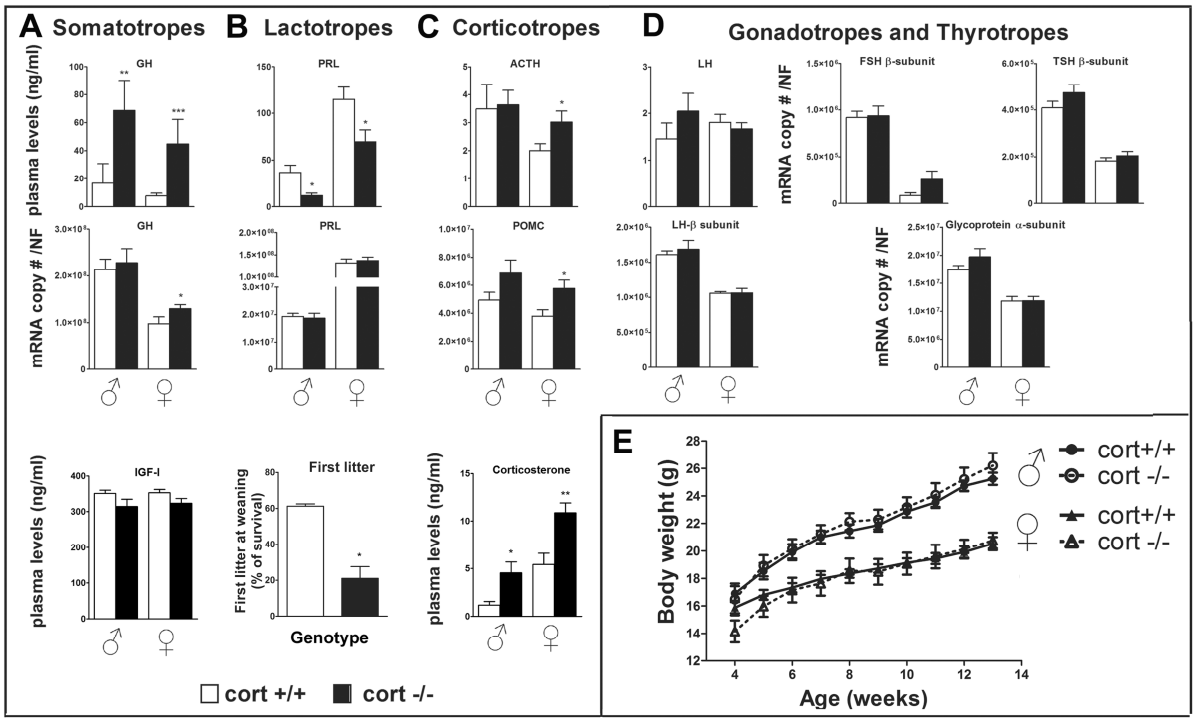

Figure 1. Regulation of pituitary somatotrope, lactotrope, corticotrope, gonadotrope and thyrotrope cell axes in cort-/- mice. A) Basal growth hormone (GH) release (top), GH expression (middle) and circulating insulin like growth factor type-I (IGF-I) levels (bottom) of male and female wild-type (cort+/+, open columns) vs. cort-/- (solid columns) mice. **B)** Basal prolactin (PRL) release (top) and PRL expression (middle) of male and female cort+/+ (open columns) vs. cort-/- (solid columns) mice. Percentage of survival of the first litter of cort+/+ (open column) vs. cort-/- (solid column) female mice (bottom). **C)** Basal adrenocorticotropin hormone (ACTH) release (top), proopiomelanocortin (POMC) expression (middle) and circulating corticosterone levels (bottom) of male and female cort+/+ (open columns) vs. cort-/- (solid columns) mice. **D)** Basal luteinizing (LH) release (top, left), β-subunit of LH expression (bottom, left), β-subunit of follicle-stimulating hormone (FSH; middle) expression, β-subunit of thyroid-stimulating (TSH) expression (top, right) and glycoprotein α-subunit expression of male and female cort+/+ (open columns) vs. cort-/- (solid columns) mice. **E)** Growth curves from males and females cort+/+ (solid lines) and cort-/- (dotted line) littermates from 4 to 13 weeks of age. Values represent mean ± SEM (6-7 mice/genotype/gender) of hormonal circulating levels or absolute hormone mRNA copy numbers [adjusted by normalization factor (NF)]. Percentages of litter survival from female mice are expressed as mean ± SEM (n=11-37 breeders). Asterisks indicate values that significantly differ from their controls (cort+/+) (*, p<0,05; **, p<0,01).

PRL output between genotypes were not associated to changes in PRL synthesis, pituitary size or relative abundance of lactotrope cells, as assessed by qrtRT-PCR, gross morphology, and by the proportion of PRL immunopositive cells, respectively (Fig. 1B and table-1). In addition, as previously reported [43] we found that male and female homozygous cort-/- were healthy and fertile. Specifically, comparison of the days to conception, days of gestation and litter size of cort-/- female did not differ from that of cort+/+ female mice (table-3). However, the percentage of cort-/- females that successfully cared for their first litter until weaning was drastically reduced as compared to cort+/+ female mice (Fig. 1B) which, would be in line with our findings showing that PRL levels are impaired in cort-/- as compared with control mice. Moreover, this percentage was not observed when compared their second litter (table 3). Pup weight at weaning (3-4 weeks of age) did not differ between cort-/- and controls [male cort +/+ 8.94 ± 0.34 vs. cort-/- 8.04 ± 0.38 g/day; female cort +/+ 8.50 ± 0.41 vs. cort-/- 8.70 ± 0.31 g/day (p=0.28)].

- *Corticotropes* (Fig. 1C): Circulating ACTH levels were markedly elevated in female but not male cort-/- mice, as compared to control animals. This increase may be related to the up-regulation of pituitary POMC mRNA levels observed only in female cort-/- mice (Fig. 1C). Differences in ACTH output between female cort-/- and cort+/+ mice were not associated with changes in pituitary size or in the relative abundance of corticotrope cells, as assessed by gross morphology and by the proportion of POMC immunopositive cells, respectively (table-1). On the other hand, circulating corticosterone levels were higher in both male and female cort-/- as compared to wild type-controls (Fig. 1C).

Table 1. Proportion of immuno-positive growth hormone (GH), prolactin (PRL), adrenocorticotropin hormone (ACTH), thyroid-stimulating hormone (TSH) and luteinizing hormone (LH) cells from male and female cort+/+ and cort-/- mice (n=4 mice/genotype/gender).

	Males				Females			
	cort+/+		cort-/-		cort+/+		cort-/-	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Somatotrophs	55,92	± 2,01	56,70	± 2,10	44,70	± 1,82	44,57	± 1,16
Lactotrophs	18,62	± 0,92	20,76	± 1,29	21,35	± 2,33	23,01	± 1,19
Corticotrophs	11,41	± 1,34	14,11	± 2,24	9,85	± 1,16	11,86	± 1,40
Thyrotrophs	6,78	± 0,65	6,64	± 0,57	5,71	± 0,46	5,76	± 0,80
Gonadotrophs	8,10	± 0,76	8,69	± 0,94	6,72	± 0,51	5,69	± 0,22

Values represent the mean ± SEM (3-4 pictures/immuno-histochemistry/genotype/mice) where the proportions shown were derived from counting more than 1000 cells/slide for each pituitary hormone tested.

- *Gonadotropes and Thyrotropes* (Fig. 1D): CST deficiency did not alter pituitary expression of β-subunits of LH, FSH and TSH or the glycoprotein hormone α-subunit and did not modify the appearance or proportion of gonadotrope or thyrotrope cells (Fig. 1D and table-1). Functional integrity of gonadotropic axis is further supported by the fact that circulating levels of LH (Fig. 1D), testosterone and estradiol (table-2) did not differ between male and female cort-/- and cort+/+ mice and by the fact that homozygous cort-/- were fertile (see above). Due to limitation of serum samples, we were unable to measure circulating FSH and TSH levels at this time. Nonetheless, these results suggest that the reproductive and thyroid axis was not altered in cort-/- mice.

Table 2. Body weight, organs weight, metabolic hormones and glucose levels in ad libitum-fed male and female cort-/- mice vs. littermate controls (cort+/+).

	Males				Females			
	cort+/+		cort-/-		cort+/+		cort-/-	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Body weight (g)	26,61 ± 0,57		25,87 ± 0,66		20,64 ± 0,44		20,01 ± 0,25	
Visceral fat (g)	0,44 ± 0,03		0,43 ± 0,04		0,33 ± 0,04		0,25 ± 0,02	
Subcutaneous fat (g)	0,29 ± 0,02		0,25 ± 0,02		0,20 ± 0,03		0,16 ± 0,03	
Liver (g)	1,13 ± 0,05		1,08 ± 0,06		0,95 ± 0,04		0,88 ± 0,01	
Pancreas (g)	0,12 ± 0,02		0,13 ± 0,02		0,10 ± 0,00		0,09 ± 0,00	
Testosterone (ng/ml)	2,55 ± 1,07		4,06 ± 2,37		n.m ±		n.m ±	
Estradiol (pg/ml)	n.m ±		n.m ±		11,54 ± 1,79		11,43 ± 2,69	
IGF-I (ng/ml)	351,68 ± 8,52		315,11 ± 19,91		353,66 ± 9,09		323,49 ± 13,35	
Corticosterone (ng/ml)	1,15 ± 0,34		4,55* ± 1,17		5,45 ± 1,17		10,89** ± 1,00	
Leptin (ng/ml)	4,08 ± 0,34		4,49 ± 0,42		3,27 ± 0,28		4,06 ± 0,30	
Glucose; fed (mg/dl)	147,42 ± 4,43		159,82 ± 6,68		129,27 ± 3,27		134,75 ± 5,28	
Glucose; fasted (mg/dl)	121,67 ± 9,67		137,75 ± 7,77		101,30 ± 8,61		118,13 ± 12,16	

Values represent the mean ± SEM of 5-8 mice/genotype/gender. Asterisks (*, p<0,05; **, p<0,01) indicate values that differ from cort +/+ within gender.

Effect of genotype on central and systemic components involved in the regulation somatotrope, lactotrope, and corticotrope funtion: metabolic profile of the cort-/- mice

To ascertain whether the gender- and CST-deficiency-dependent changes observed in pituitary hormone expression and/or release of cort-/- mice could be related to changes in hypothalamic or systemic inputs, we analyzed and compared the expression and/or circulating levels of the main regulators of the

corresponding somatotrope, lactotrope, and corticotrope cell functions in male and female wild type-controls and cort-/- mice.

- *Hypothalamic phenotype*: Expression levels of various regulatory systems of pituitary cell function, including GH-releasing hormone (GHRH), SST and its receptors (sst1-5 or truncated sst5-variants), PRL-releasing hormone (PRLRH), corticotrope-releasing factor (CRF), urocortins (Ucn-2 and -3), gonadotrope-releasing hormone (GnRH), POMC, neuropeptide Y, Kiss-1, thyrotropin-releasing hormone (TRH) or ghrelin, In2-ghrelin variant, GHS-R and ghrelin O-acyl transferase (enzyme responsible of ghrelin acylation; GOAT) were not altered in male or female cort-/- compared with their cort+/+ mice counterparts (supplemental table-2) which lessen the potential involvement of a central, hypothalamic regulation in the changes observed in the pituitaryof cort-/- mice.

- *Pituitary phenotype* (Fig. 2): SST expression was not altered in pituitary of cort-/- mice however, we observed a striking sexual dimorphism in the mRNA levels for ssts subtypes (Fig. 2A). Specifically, an overall elevation in pituitary ssts-subtypes mRNA levels in female cort-/- compared with control mice was observed, which reached statistical significance for sst2A, sst3, sst5 and sst5TMD1. Expression levels of GHRH-R, ghrelin, In2-ghrelin, GOAT, GHS-R (Fig. 2B) or CRF receptors (CRF-R1 and CRF-R2; Fig. 2B) did not differ between male or female cort-/- and cort+/+. In contrast, dopamine-receptor type-2 (DR2) and PRLRH-receptor mRNA levels were up-regulated in the pituitary of female cort-/- mice compared with cort+/+ animals (Fig. 2C).

Table 3. Fertility endpoints in female cort -/- mice, compared to female cort +/+ mice in a C57Bl/6J background.

	Female breeder			
	cort+/+		**cort-/-**	
	Mean	*SEM*	*Mean*	*SEM*
Days to conception (d)	6,11 ± 2,40		4,70 ± 0,90	
Days of gestation (d)	20,57 ± 0,20		20,00 ± 0,33	
Litter size (n° of pups)	5,06 ± 0,39		4,96 ± 0,43	
First litter at weaning (%)	61,2		21,1	
Second litter at weaning (%)	50		64,6	
	Pairs tested (n=11-37)			

Values represent the mean ± SEM of 11-37 female breeder mice/genotype. Asterisks (*, p<0,05; **, p<0,01) indicate values that differ from cort +/+.

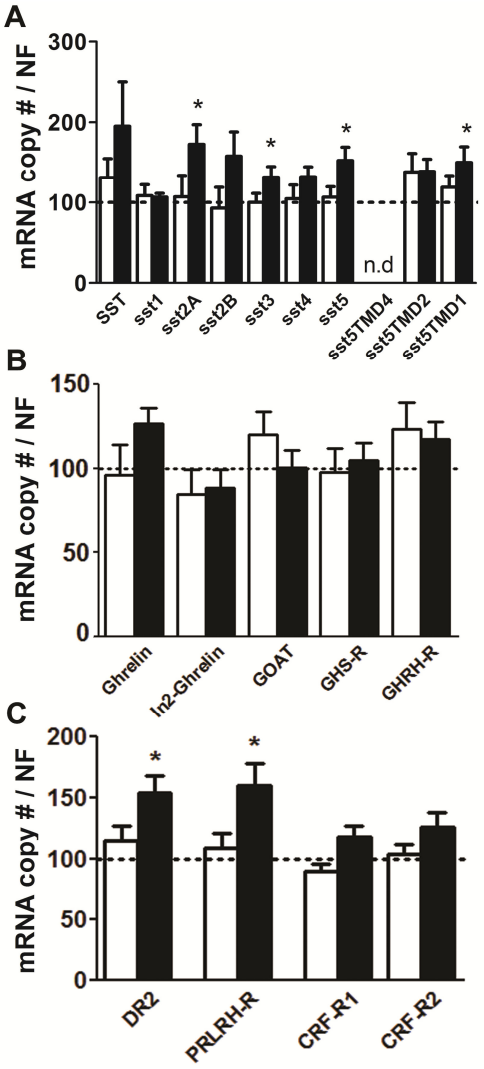

Figure 2. Expression of pituitary components involved in the regulation of somatotrope, lactotrope and corticotrope axes in cort-/- and cort+/+ mice. A) Expression of SST and SST receptors isoforms/variants (ssts), **B)** Expression of ghrelin, In2-ghrelin, ghrelin O-acyl tranferase (GOAT), ghrelin receptor (GHS-R) and growth hormone releasing hormone receptor (GHRH-R) and, **C)** Expression of dopamine receptor 2 (DR2), prolactin releasing hormone receptor (PRLRH-R) and corticotropin releasing factor receptor type 1 and 2 (CRF-R1,2) in male (open columns) and female (solid columns) cort-/- mice. Values are showed as relative percentage of male or female cort-/- vs. control (cort+/+) mice (shown by the dotted line set at 100%) and represent the mean ± SEM of 5-8 mice/gender. Asterisk (*, p<0,05) indicates values that significantly differ from cort +/+ within gender.

- *Stomach phenotype*: Plasma levels of acylated as well as total (acylated + unacylated) ghrelin were clearly elevated in female cort-/- mice as compared to their cort +/+ controls whereas no such differences were observed in males (Fig. 3A, left panel). This pattern compared well with, and is likely supported by, the markedly increased levels of expression of ghrelin and GOAT observed only in female cort-/- mice with respect to their controls (Fig. 3B, left panel). In contrast with these gender-dependent changes, mRNA levels for another

gastric hormone, gastrin, did not differ between genotypes (supplemental table-2). Also, in partial contrast with these findings, circulating total (but not acylated) ghrelin levels were up-regulated in both male and female sst-/-, as compared with sst+/+ mice (Fig 3A, right panel). Moreover, expression of ghrelin was up-regulated in male, but not female, sst-/- mice while GOAT mRNA levels were not altered in sst-/- across genders as compared with sst+/+ mice (Fig. 3B, right panel).

Interestingly, stomach SST expression was highly up-regulated in female cort-/- mice, whereas mRNA levels of sst-subtypes were similar in cort-/- and cort+/+ mice across genders, which is in clear contrast with that previously reported in sst-/- mice [59]. The increase in stomach SST expression observed in female cort-/- was likely translated into SST release, since we found that circulating SST levels tended (p=0.07) to be elevated in female, but not male cort-/- as compared with cort+/+ mice (Fig. 3D).

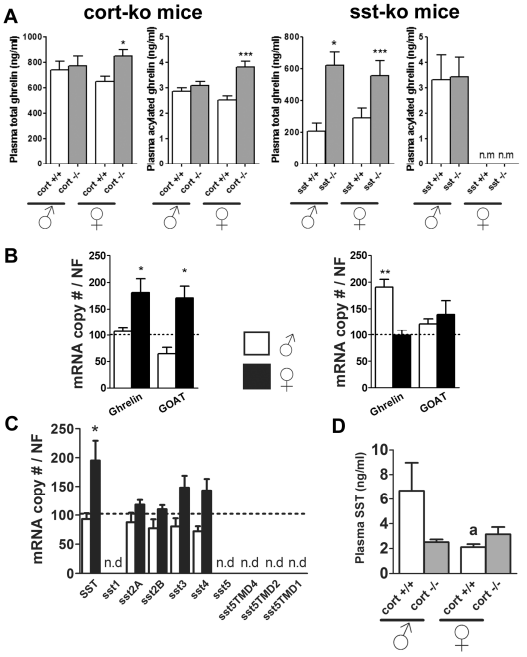

Figure 3. Impact of lack of endogenous CST or SST in the regulation of stomach somatostatin and ghrelin systems. A) Circulating levels of acylated and total (acylated plus non-acylated) ghrelin in male and female cort+/+ (white columns) vs. cort-/- (grey columns) mice (left) and sst+/+ (white columns) vs. sst -/- (grey columns) mice (right). **B)** Stomach expression of ghrelin and ghrelin O-acyl tranferase (GOAT) of male (open columns) and female (solid columns) cort+/+ vs. cort-/- mice (left) and male (open columns) and female (solid columns) sst+/+ vs. sst-/- mice (right). **C)** Stomach expression of SST and SST receptors isoforms/variants (ssts) of male (open columns) and female (solid columns) cort+/+ vs. cort-/- mice. **D)** Circulating SST levels in male and female cort+/+ (white columns) vs. cort-/- (grey columns) mice. Circulating hormone levels (panels A and D) are represented as the mean ± SEM of male and female cort-/- or sst-/- vs. littermate-controls (cort+/+ or sst+/+, respectively; 4-10 mice/genotype/gender). Expression levels (panels B and C) are showed as relative percentage of male or female cort-/- vs. control (cort+/+) mice (shown by the dotted line set at 100%) and represent the mean ± SEM of 5-8 mice/genotype/gender. Asterisks (*, p<0,05; **, p<0,01; ***, p<0,001) indicate differences between controls (+/+) and knockout (-/-) mice within gender.

- *Liver phenotype*: Despite changes in circulating GH, there were no differences in plasma IGF-I levels (Fig. 1A) or in the expression levels of acid-labile subunit (ALS), IGF-I or IGF-I variants 1-5 (supplemental table-2) between male or female cort-/- and cort+/+ mice. Likewise, mRNA levels of other GH-regulated hepatic transcripts (GH-R, PRL-R or MUP-3) did not differ between genotypes (supplemental table-2).

- *Pancreas phenotype* and effect of genotype on glucose homeostasis: Circulating insulin levels were markedly reduced in male and female cort-/- mice as compared with their wild type littermates (Fig. 4A, top panel), whereas plasma levels of glucagon were not altered by CST deficiency (Fig. 4A, bottom panel). Interestingly, despite the disparate insulin levels, glucose levels in male and female cort-/- mice were similar to those of their cort+/+ mice counterparts under both normal-fed and fasted (overnight) conditions though a small, non-significat trend for glucose levels to be higher was noted in fed (p=0.14) male cort-/- mice (table-2).

To further evaluate the effect of CST deficiency on glucose homeostasis, GTT and ITT were performed in cort-/- and cort+/+ mice, and were compared with those of sst-/- and sst+/+ mice (Fig. 4B-C and 4D-E, respectively). This revealed that male cort-/- mice have an overall impairment in insulin-mediated glucose clearance (p=0.015; Fig. 4B top/left panel) as compared with cort+/+ mice, even though there was no statistical differences at any of the individual time points tested of the GTT, and in the global AUC measure (Fig. 4C, top panel), cort-/- male mice only showed a non-significant tendency (P=0.23) to be insulin resistant as compared with cort+/+ mice (male AUC controls 29068 ± 1885 vs. male cort-/- 32933 ± 2506 mg/dl). In clear contrast, GTT response in female mice was fully undistinguishable across genotype (Fig. 4B top/right-panel and 4C top-panel; AUC controls 22857 ± 1473 vs. female cort-/- 23657 ± 1319 mg/dl).

Interestingly, analysis of the response to ITT further unveiled a CST deficiency-dependent deterioration of glucose homeostasis in male mice, as glucose levels remained significantly lower in cort+/+ male mice as compared with their cort-/- littermates at both 60 and 120 min post-insulin injection (Fig. 4B bottom left-panel) and cort-/- male mice also showed an overall impairment of insulin-mediated glucose clearance (Fig. 4C, bottom-panel; AUC controls 7388 ± 846 vs. cort-/- 11467 ± 1407 mg/dl, p=0.017). Again, this was in marked contrast with the nearly identical ITT response observed in female cort-/- and cort+/+

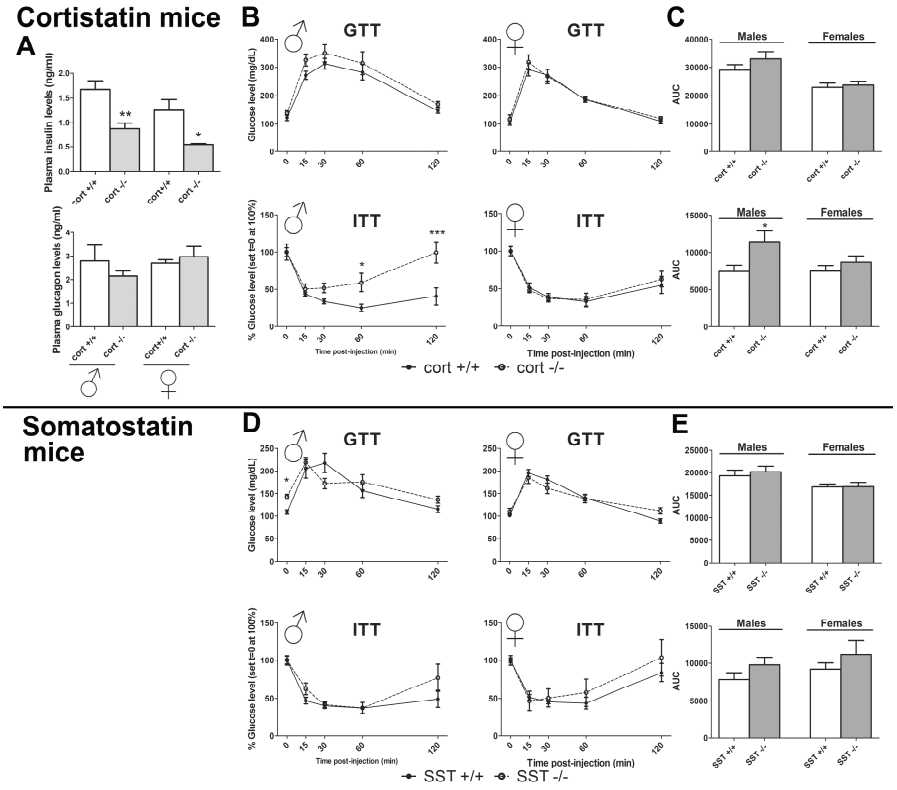

Figure 4. Impact of lack of endogenous CST or SST on glucose homeostasis. A) Circulating insulin (top) and glucagon (bottom) levels in male and female cort+/+ (white columns) vs. cort-/- (grey columns) mice. **B)** Glucose tolerance tests (GTT, top) and insulin tolerance tests (ITT, bottom) of male (right) and female (right) cort+/+ (solid lines) vs. cort-/- (dotted lines) mice. **C)** Area under curve (AUC) of GTT (top) and ITT (bottom) conducted in male and female cort+/+ (white columns) vs. cort-/- (grey columns) mice. **D)** GTT (top) and ITT (bottom) of male (right) and female (right) sst+/+ (solid lines) vs. sst-/- (dotted lines) mice. **E)** AUC of GTT (top) and ITT (bottom) conducted in male and female sst+/+ (white columns) vs. sst-/- (grey columns) mice. Values are represented as the means ± SEM (n=6-12 mice/genotype/gender). Asterisks (*, p<0,05; **, p<0,01; ***, p<0,001) indicate values that significantly differ from cort+/+ (A, B, C) or sst+/+ (D, E) within gender.

identical ITT response observed in female cort-/- and cort+/+ mice (Fig. 4B bottom/right-panel and 4C bottom-panel; AUC controls 7438 ± 767 vs. cort-/- 8661 ± 863 mg/dl, p=0.3).

At variance with that found for CST, absence of SST did not seem to relevantly influence the regulation of glucose homeostasis (Fig. 4D-E). Thus, no differences were found in GTT or ITT between genotypes regardless of the gender of mice. In addition, it should be noted that no statistical differences have been previously detected in SST-KO by our group [49] and other laboratories [60] in basal plasma levels of insulin or glucagon.

In vitro pituitary hormone output in the presence and absence of endogenous CST and effect of CST on primary pituitary cell cultures of mouse and primate

To further examine the functional and physiological relevance of the effects caused by CST deficiency, we employed in vitro models of pituitary cell cultures from mice and primate. This showed that, consistent with the in vivo data obtained in cort-/- mice, CST did not alter the expression of PRL, β-subunits of LH, FSH and TSH or glycoprotein hormone α-subunit in primary pituitary cultures of normal male or female mice (Fig-5A). However, CST inhibited GH and POMC mRNA levels in pituitary cells from normal female mice but not in those from male mice (Fig-5A). Moreover, in pituitary cell cultures from both male and female mice, CST significantly suppressed the release of GH and ACTH while it increased PRL secretion (Fig-5B). Of note, the decreased levels of circulating PRL levels observed in vivo in cort-/- mice of both genders and the elevated ACTH levels observed only in female cort-/- mice in vivo were similarly maintained in vitro, under basal conditions in the corresponding pituitary cell cultures (Fig-5C). In contrast, basal GH release levels were similar between primary pituitary cell cultures of cort-/- and cort+/+ (Fig-5C). In line with these results, our data provides strong evidence that CST can directly acts on mouse primary pituitary cell cultures by increasing or decreasing [Ca2+]i (table 4), a well-known, pivotal second messenger directly and necessary linked to hormone release [61, 62]. Therefore, these results reinforce the idea of the existence of two populations of pituitary cells that differentially respond to CST, one negatively (probably somatotropes and/or corticotropes) and another positively (probably lactotropes).

Table 4. Percentage of primary cultured pituitary cells showing positive or negative changes in [Ca2+]i in response to CST-14 (100nM) in female mice.

	Cells	% Max SEM	Time SEM
↑ [Ca2+]i	12%	140,47 ± 9,82	41,11 ± 7,49
↓ [Ca2+]i	16%	77,69 ± 3,72	40,00 ± 6,42

Percentage of maximum response (%Max) and time of response to CST administration are also indicated. (n=75, cort+/+).

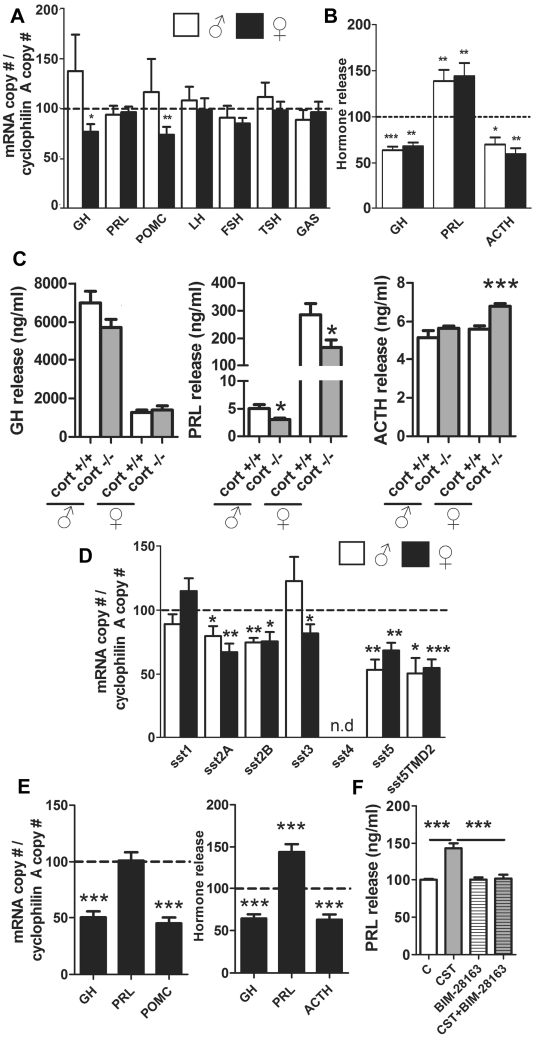

Figure 5. Direct effect of CST or lack of endogenous CST on pituitary hormonal expression and secretion of male/female mice and female baboons. A) Effect of CST-14 (100nM, 24h) on growth hormone (GH), prolactin (PRL), proopiomelanocortin (POMC), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and glycoprotein alfa-subunit (GAS) expression on primary pituitary cell cultures of male (white columns) and female wildtype (cort+/+) mice. **B)** Effect of CST-14 (100nM, 24h) on GH, PRL and adrenocorticotropin hormone (ACTH) release on primary pituitary cell cultures of male (white columns) and female (black columns) wildtype (cort+/+) mice. **C)** GH, PRL and ACTH release levels (24h culture; ng/ml) in primary pituitary cell cultures of male and female cort+/+ (white columns) and cort-/- (grey columns) mice under basal condition. **D)** Effect of CST-14 (100nM, 24h) on SST receptors isoforms/variants (ssts) on primary pituitary cell cultures of male (white columns) and female (black columns) wildtype (cort+/+) mice. **E)** Effect of CST-17 (100nM, 24h) on GH, PRL and POMC expression (left) and on GH, PRL and ACTH release in primary pituitary cell cultures of female baboon. **F)** Effect of CST-17 (100nM, 24h) alone or in combination with BIM-28163 (GHS-R1a antagonist; 10nM) on PRL release in primary pituitary cell cultures of female baboon [vehicle-treated control (C) was set at 100%]. Values are represented as the means ± SEM of 3-4 independent experiments (3-5 wells/treatment/genotype/gender) and, in figures A, B, D and E are expressed as percentage of vehicle-treated controls (shown by the dotted line set at 100%). Asterisks (*, p<0,05; **, p<0,01; ***, p<0,001) indicate values that differ from the corresponding controls within genders (from vehicle-treated controls for figures A, B, D and E; from cort+/+ within genders for figure C or from vehicle-treated controls or CST-treated cells for figure F).

CST treatment did not alter sst1 expression in pituitary cell cultures, whereas it decreased sst2A, sst2B, sst5, and sst5TMD2 in both male and female mice and decreased sst3 only in primary pituitary cell cultures from females (Fig.-5D).

In line with that found in mice, in vitro treatment with CST inhibited the spontaneous secretion of GH and ACTH and decreased GH and POMC mRNA levels in primary pituitary cell cultures from female baboons (Fig-5E, left-panel). Moreover, the same CST treatment stimulated baboon PRL release while it did not alter PRL expression (Fig-5E, right-panel). Of note, CST-stimulated PRL release was completely blocked by an antagonist of GHS-R1a (Fig. 5F), suggesting that the stimulatory effect of CST on baboon PRL release was directly mediated via ghrelin-receptor.

Discussion

Based on the remarkable structural similarity of CST to SST, their close sst-binding profiles and a limited set of in vivo and in vitro results, it has been implicitly assumed in the past that CST is a mere SST analog regarding its endocrine and metabolic actions. This overly simplistic assumption is challenged by the results obtained in the present study, wherein we have performed, for the first time, a thorough characterization of the endocrine-metabolic profile of cort-/- mice at the hypothalamic, pituitary and systemic levels, and have analyzed in detail the direct effects that CST exerts on hormone synthesis and/or release from pituitary cells in male and female mice, as well as in a normal non-human primate model.

Given its expected relevance as a CST target, the somatotrope cell was the first pituitary cell type analyzed, along with its accompanying axis. This revealed that circulating GH levels are markedly elevated in both male and female cort-/- mice as compared to their cort+/+ controls, thereby indicating that CST is a plausible relevant player in the physiological regulation of GH secretion. However, consistent with that previously reported for SST-KO mice [46, 49, 58], elevated plasma GH levels observed in cort-/- mice did not result in significant changes in IGF-I levels, or in increased body weight or linear growth, suggesting that CST absence is not a critical factor in the control of GH-induced somatic growth, at least under the conditions examined herein (KO). Likewise, as in SST-KO mice [49], elevated plasma GH levels in cort-/- mice of both genders were not accompanied by changes in the proportion of somatotrope cells with respect to cort+/+ mice, suggesting an increased GH secretory activity of individual somatotropes in CST-KO mice. However, at variance with that found in SST-KO

mice [49], the enhanced GH release observed in cort-/- mice in vivo was not maintained in vitro. This, coupled with our observation that CST is not expressed at the pituitary level in control mice (cort+/+; data not shown), suggests that circulating CST arising from other tissue sources should account for the changes in GH-output observed in vivo.

Interestingly, our findings unveiled unique, gender-dependent actions for CST on the GH axis, which are not reproduced in SST-KO mice, and are likely linked to other GH-related functions (see below). Indeed, the higher enhancement of GH release in female (six-fold) than in male (four-fold) cort-/- mice with respect to their normal littermates led to similarly elevated plasma GH levels, and thereby blunted the typical gender-dependent difference in circulating GH, which was observable in cort+/+ mice. This suggested that female somatotropes are more sensitive to the absence of endogenous CST in vivo. In fact, expression levels of GH, as well as those of most sst-subtypes (sst2A/B, sst3, sst5 and sst5TMD1, which are highly expressed in GH-cells (our unpublished observations) and account for ~60% of total sst transcripts in pituitary [59] were elevated in pituitaries of female, but not male cort-/- mice as compared with their littermate controls (cort+/+).

A more detailed analysis of the impact of CST on somatotrope function using primary pituitary cell cultures give credence to the notion that lack of endogenous CST would directly account, at least in part, for the changes observed in the GH-axis of cort-/- mice in vivo, and specially for its gender-dependent implications. Thus, CST treatment inhibited GH release in pituitary cells from both male and female mice as well as from baboons. Furthermore, our findings revealed, for the first time in any species, that CST also reduces GH mRNA levels, yet it only did so in cultures from female mice (and female baboons) and not in pituitary cells derived from male mice. The ability of CST to inhibit GH release observed herein in mice and baboons is consistent with earlier reports showing that CST inhibits GH release in vitro in human fetal pituitary cell cultures and in ~50% of the GH-secreting pituitary adenoma cultures analyzed [63], as well as in vivo, in healthy young human male [23], in patients with active GH-secreting adenomas [23, 24], and in normal male anaesthetized rats [64]. In contrast, other reports that analyzed primary pituitary cell cultures from female pigs [65] and frogs [66] or GH-secreting adenomas cultures [63] have shown that CST [or "Pro2, Met13 somatostatin-14" (SS2), the CST counterpart in frogs] did not significantly affect basal GH release. Moreover, controversial observations have also been reported in primary pituitary cell cultures from male rats [67] and female pigs [65] and in 20% of cases of GH-secreting adenomas cultures [24], where CST, depending of the dose applied, can increase GH release. These disparate findings unveil the partial, incomplete understanding gathered hitherto on the direct pituitary actions of CST in controlling GH secretion, which has largely been based on studies conducted in non-primate species (either male or female) or in primary cell cultures from human fetal pituitaries or GH-producing adenomas. In this scenario, our present report provides compelling evidence for a relevant role of CST in the control of the somatotrope function since: 1) lack of endogenous CST (cort-/-) evokes a potent increase in circulating GH levels in both male and female mice in vivo, whereas it only increases GH expression in female mice; 2) these in vivo results are fully supported by the first simultaneous comparison of the in vitro effect of CST on somatotrope function (GH synthesis and release) in both genders of a non-primate species (male and female mice); and 3) by the demonstration of a direct, major inhibitory action of CST on GH secretion and/or synthesis in a normal non-human primate (baboons) model, likely relevant for normal human physiology [50, 51, 68-70].

We next explored whether changes in the expression of other factors known to influence somatotrope function at different levels (hypothalamic, pituitary, and/or systemic; [71-74]), besides CST itself, could also be involved in the altered somatotrope function observed in cort-/- mice. Our results revealed that changes in GH synthesis and release found in cort-/- mice were independent of changes in hypothalamic expression of SST, GHRH, pituitary GHRH-R or hypothalamic or pituitary ghrelin-system (ghrelin, In2-ghrelin, GOAT, and GHS-R). In contrast, as mentioned above, an overall up-regulation in the expression of pituitary sst-subtypes (sst2A/B, sst3, sst5 and sst5TMD1) was found in female, but not male cort-/- mice, which could be interpreted as a compensatory mechanism to decrease the particularly elevated levels of GH synthesis and release of female cort-/- mice. Interestingly, examination of systemic/peripheral signals revealed changes in cort-/- mice of both genders that would favor elevated GH release, such as up-regulated glucocorticoid levels and down-regulated insulin levels. Of note, an up-regulation of stomach ghrelin and GOAT mRNA levels was observed only in females, which likely supports the subsequent elevation in circulating total- and acylated-ghrelin levels observed exclusively in female, not in male cort-/- mice. In fact, this may contribute, in turn, to the enhanced sensitivity of the female GH-axis to CST loss (i.e.

significant up-regulation of GH synthesis and release as compared with male). Finally, it is worth noting that, in spite of the elevated plasma GH levels observed in cort-/- mice, circulating IGF-I levels, liver weight or the expression of hepatic genes sensitive to changes in GH levels [75], did not vary between cort-/- mice and their controls (cort+/+) regardless of gender. Inasmuch as these changes are, at least in part, substantially different to those observed in SST-KO mice [49, 58], it seems reasonable to propose that the physiological mechanisms involved in the regulation of GH-axis by CST and SST mice should differ (at central, pituitary and/or systemic levels) and therefore, that CST is not a mere natural analogue of SST in regulating gender-dependent, metabolic/endocrine GH-related secretions.

In line with the above notion, insulin levels in male and female cort-/- mice were found to be decreased compared with their cort+/+ controls under fed conditions albeit this was not associated with an improvement in the response to ITT. In fact, the responses to glucose and insulin (GTT and ITT) were deteriorated in male cort-/- mice compared with their cort+/+ littermates, unveiling an overall impairment in insulin-mediated glucose clearance, while GTT and ITT responses in female cort-/- mice, as in male and female SST-KO, were similar to their respective controls. These latter results compare well with those of the only previous report on male SST-KO mice in response to glucose challenge [60]. The interpretation of our findings may not be immediately apparent. It has been demonstrated that simultaneous elevation in circulating levels of GH and glucocorticoids (as seen in cort-/- and SST-KO of both genders) [present study and [46, 47]] could cause insulin resistant [76, 77]. However, it should be noted that only male cort-/- mice were found to be insulin-resistant, thus raising the question as to which factors could underlie the gender- and genotype-dependent differences observed in cort-/- and SST-KO mice. We hypothesized that the elevated circulating levels of ghrelin found in male and female SST-KO as well as in female cort-/- mice could have a protective role in pancreatic function, because the non-acylated form of ghrelin has been previously shown to exert a favorable influence on insulin sensitivity and glucose homeostasis [78-80], where circulating total-ghrelin levels were found to be up-regulated in SST-KO of both genders [47, 49, 81] and in female but not male cort-/- mice. Taken together, our results disclose a previously unknownz(albeit suspected [82]) involvement of CST in the control of insulin/glucose homeostasis, which may be physiologically relevant, differs from the related actions of SST, and shows a gender divergence that may possibly entail a distinct participation of the ghrelin system in male and female mice.

In contrast to the expectable influence of CST on the GH axis, evidence on the possible relationship between CST and prolactin was scarce. It was thus surprising that circulating PRL levels were markedly down-regulated (~50%) in both, male and female cort-/- mice as compared to their cort+/+ controls. This decrease was not associated to any changes in the proportion of lactotrope cells or PRL expression in pituitary, suggesting that a decreased PRL-secretory capacity of individual lactotropes underlies the decreased plasma PRL levels of cort-/- mice. This idea is further supported by the reduced PRL release observed in vitro under basal conditions in primary pituitary cells derived from both, male and female cort-/- with respect to their cort+/+ controls. This observation uncovers a completely novel idea, namely, that endogenous CST exerts a positive role on PRL output, and that the absence of endogenous CST input may have changed permanently the internal programming of lactotropes. These results were unexpected based on the limited information available, which indicates that both CST and SST inhibit PRL release in vivo in patients with prolactinomas [24] but not in healthy human men [23, 24, 82], and that CST inhibits PRL release in vitro in cultured prolactinomas [63], similar to SST, which inhibits PRL release in cultured pituitary cells from fetal human, rat and fish [83-85]. Nevertheless, our findings are strongly supported by results from a recent study showing that CST increases PRL release in vivo in male rats [86]. Besides, there was indirect evidence that CST could enhance PRL release in humans, since ghrelin-induced PRL increase in normal human subjects was significantly higher when CST was co-administered simultaneously (unfortunately, effects of CST treatment alone was not shown in this study [23]. Our findings led to the question of what are the direct (pituitary) components and precise mechanism mediating the actions of CST on lactotrope function. To ascertain this issue, primary pituitary cell cultures of male and female mice and female baboons were challenged with CST, which clearly increased PRL release (but not expression) in both mice and baboons, thus supporting the results obtained with cort-/- mice in vivo. These results are in striking contrast with the inhibitory effect exerted by SST on PRL secretion in the same baboon pituitary cell cultures (data not shown), specially in light of the highly similar binding profile of SST and CST for the family of sst receptors [53]. Accordingly, it was reasonable to think that the unique stimulatory effect of CST on PRL release

should be exerted through a different receptor, not shared by SST. Interestingly, it has been reported that in human pituitary CST, but not SST, binds with high affinity to GHS-R1a, the receptor for ghrelin [31, 41], and this peptide, in turn, is known to stimulate PRL secretion [23, 87, 88]. Therefore, we sought to determine whether CST-induced PRL release could be exerted via GHSR1a. Indeed, use of a specific antagonist for GHSR1a, BIM-28163, fully blocked CST-stimulated PRL release in primary baboon pituitary cells, thereby indicating that CST acts via the ghrelin receptor to induce its stimulatory effect on PRL secretion. The potential physiological relevance of this novel PRL-stimulatory action of CST is as yet unknown. However, in support for such relevance is our finding that the percentage of cort-/- females that successfully cared for their first litter was significantly lower compared to cort+/+ female mice. Since prolactin is well-known to be required for maternal milk production and luteal maintenance, and also plays a key role in the regulation of maternal behavior [89, 90] further studies are warranted in the future aimed at clarifying the possible involvement of CST in controlling these PRL functions.

Analysis of the corticotropic axis in cort-/- mice revealed that CST could also relevantly influence its regulation. Thus, as mentioned above, circulating corticosterone levels were upregulated in male and female cort-/-, a finding that is in line with that reported for SST-KO mice [46, 47] and with prior evidence that CST reduces cortisol levels in patients with Cushing disease [91]. However, elevated glucocorticoid levels were accompanied with concomitant increases in circulating ACTH levels and pituitary POMC expression only in female, but not in male cort-/- mice compared with their controls. These elevated circulating ACTH levels in female cort-/- mice were not due to an increased proportion of corticotropes but most likely to an enhanced ACTH-secretory capacity of individual corticotropes, as indicated by the augmented ACTH release observed in primary pituitary cell cultures of female, but not male, cort-/- mice under basal conditions. In addition, the results observed in vivo were supported, at least in part, by our in vitro data showing that CST treatment decreased ACTH release from pituitary cell cultures of male and female mice and baboons, whereas it only inhibited POMC expression in female mice (and baboons). Taken together, our results suggest that endogenous CST contributes to regulate ACTH release in a gender-dependent manner. This latter divergence would not be accounted for by changes in hypothalamic expression of primary regulators of ACTH secretion (CRF or urocortins) [92] or by changes in pituitary sensitivity to these hypothalamic factors (CRF/urocortin receptors, CRF-R1 and CRF-R2). Instead, the general upregulation of the ghrelin system (acylated and total ghrelin levels and stomach ghrelin and GOAT expression) observed only in female cort-/- mice could be related to the selective increase in circulating ACTH observed in female cort-/-, since acylated ghrelin can positively regulate corticotrope function in humans [23, 82, 93], baboons [47] and other species [94, 95]. When viewed as a whole, our data unveil novel endocrine functions on the corticotropic axis for CST with plausible physiological impact, which deserve further investigation.

In summary, results of the present study on a thorough analysis of cort-/- mice, complemented with in vitro studies on mice and baboon pituitary cells provide compelling evidence that CST is not a simple natural alternate for SST but possesses unique endocrine-metabolic actions of its own, which may involve physiologically relevant functions. Given the striking similitude of SST and CST at many levels, our results underscore the renewed importance of identifying the molecular signatures and selective mechanisms of action (receptors, signals, targets) that confer the distinct functional abilities to these two peptides. In particular, the unique, unexpected actions of CST on the control of PRL, ACTH, and GH-related glucose-insulin homeostasis, and the fact that these actions, not mimicked by SST, are strongly gender-dependent offer new grounds to investigate the hitherto underestimated physiological relevance of CST in the regulation of endocrine and metabolic process. The plausible involvement of ghrelin system components in mediating some of these unique actions of CST can shed new light in the complex relationship between these molecules (CST/SST/ghrelin) in regulating multiple, common targets in health and disease.

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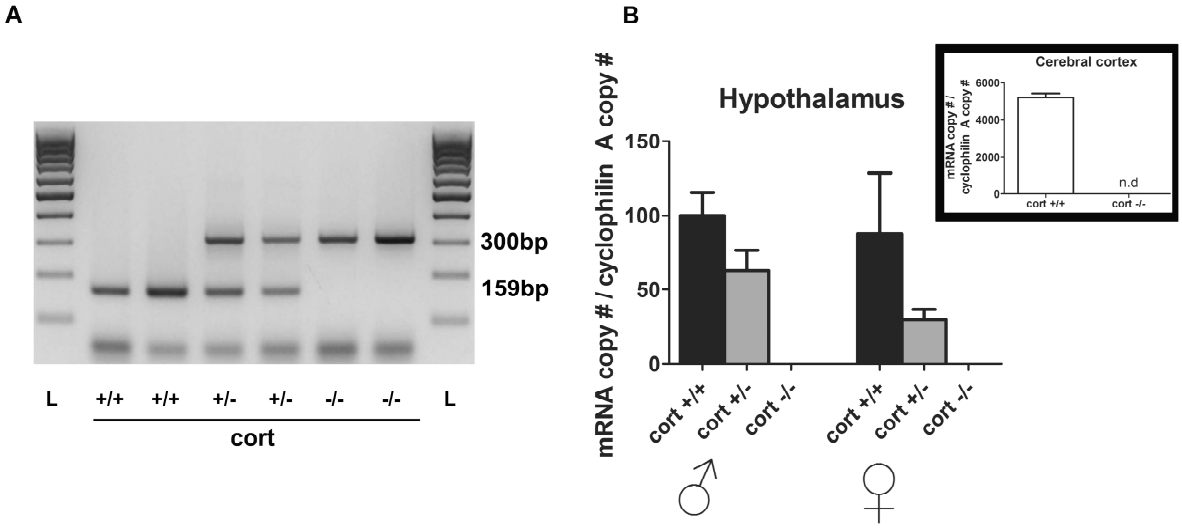
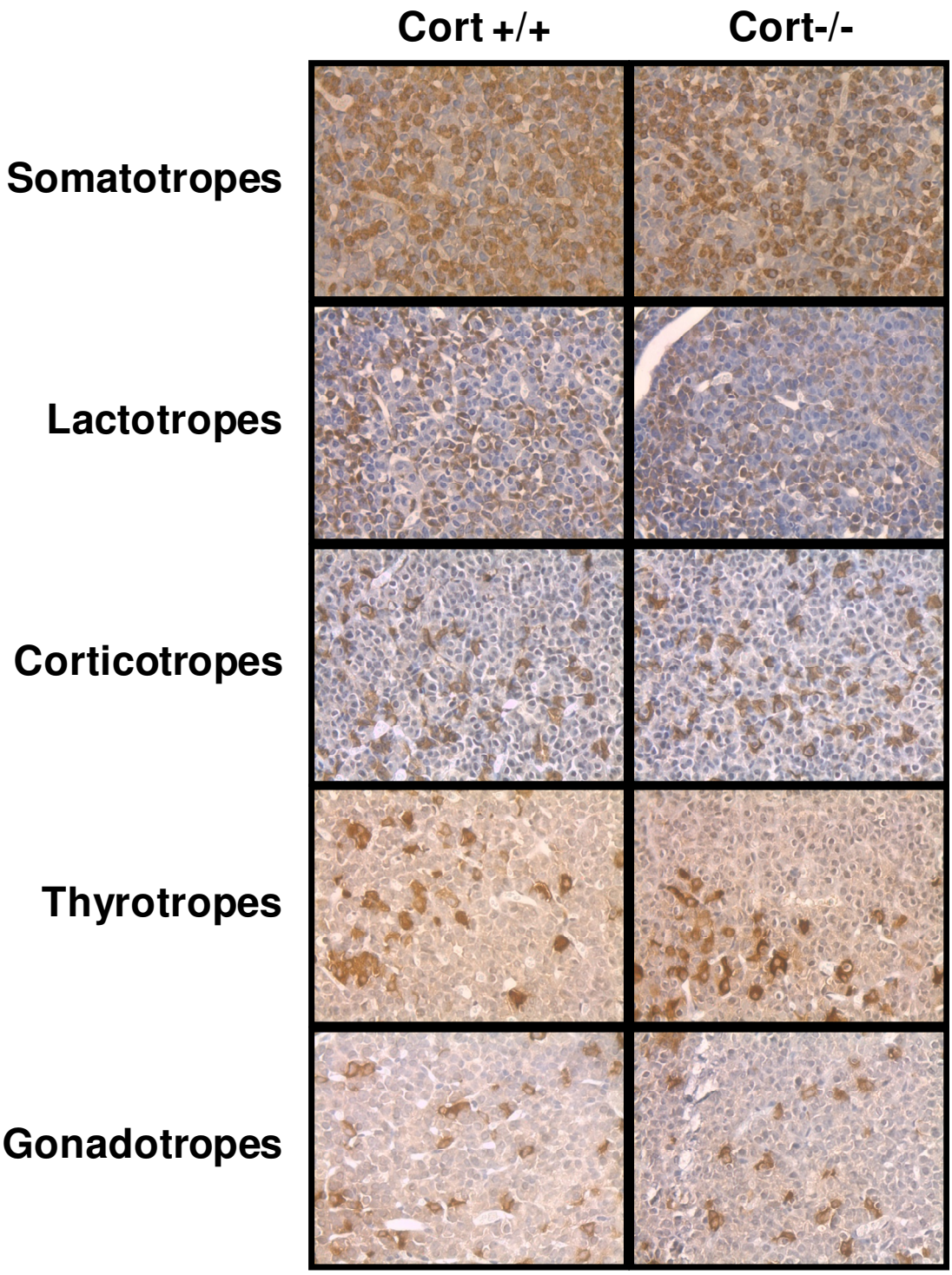


Supplemental Figure-1: Genotyping of mice and cortistatin expression levels in hypothalamus and cortex. A) Representative agarose gel showing PCR products amplified by PCR using genomic DNA from tail-snip of mice as template and primer 1-3 for CST-genotyping (see Supplemental Table 1). All products were size separated on an agarose gel containing ethidium bromide. 300bp band correspond to wild-type (+) allele PCR product and 159bp band correspond to knock-out (-) allele PCR product. Determination by size in agarose gel of only + allele, only - allele or both, allows us to determine the genotype of cort +/+, cort -/- or cort +/- mice, respectively. **B)** Analysis of CST expression in hypothalamus of male and female cort +/+, cort +/-, and cort -/- mice. In addition, CST mRNA expression was determined in cerebral cortex of male cort +/+ and cort -/-. Values are represent as the means ± SEM of 5-8 mice/genotype/gender and are expressed as absolute CST mRNA copy numbers adjusted by cyclophilin A (used as housekeeping gene).



Supplemental Figure-2: Representative micrographs (immuno-histochemistry) of impact of lack of endogenous CST on the appearance of growth hormone (GH), prolactin (PRL), adrenocorticotropin (ACTH), thyroid-stimulating hormone (TSH) and luteinizing hormone (LH) cells in male cort +/+ and cort -/- mice. Similar results were obtained with female cort +/+ and cort -/- mice as shown in table-1.

Supplemental table 1: Specific set of primers for genotyping of mouse cort-colony (cort+/+, cort+/- and cort-/-) by PCR of tail-snip DNA. Specific primers for amplification of mouse and baboon transcripts used for quantitative real-time RT-PCR.

Template	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
Genotyping cort +	AF050156	**Primer 1:** AGTGCACCTGCTCGGTTCGTCTC	**Sn** 1352	159
		Primer 2: TCCCTGACAGACCCAGGCTAGGA	**As** 1510	
Genotyping cort -	NT_039268.5[a]	**Primer 1:** AGTGCACCTGCTCGGTTCGTCTC	**Sn** 2960754	300[b]
		Primer 3: CATGTCGCACGTGCATGTCGAC	**As** 2959185	
CST	NM_007745	**Sense:** AAGAGACCCTCGTCCACCAA	**Sn** 52	213
		Antisense: ACCAGGCAAGGAAAGTCAGAAG	**As** 264	
Somatostatin and somatostatin receptors				
SST	NM_009215.1	**Sense:** TCTGCATCGTCCTGGCTTT	**Sn** 138	113
		Antisense: CTTGGCCAGTTCCTGTTTCC	**As** 250	
sst1	NM_009216	**Sense:** TGCCCTTTCTGGTCACTTCC	**Sn** 757	135
		Antisense: AGCGGTCCACACTAAGCACA	**As** 891	
sst2A	NM_001042606	**Sense:** CCCATCCTGTACGCCTTCTT	**Sn** 925	134
		Antisense: GTCTCATTCAGCCGGGATTT	**As** 1058	
sst2B	NM_009217.2	**Sense:** TGATCCTCACCTATGCCAACA	**Sn** 893	105
		Antisense: CTGCCTTGACCAAGCAAAGA	**As** 997	
sst3	NM_009218.3	**Sense:** GCCTTCTTCGGCCTCTACTT	**Sn** 1292	139
		Antisense: GAATGCGACGTGATGGTCTT	**As** 1430	
sst4	NM_009219.3	**Sense:** AGGCTCGTGCTAATGGTGGT	**Sn** 860	121
		Antisense: GGATGAGGGACACATGGTTG	**As** 980	
sst5	NM_011425.2	**Sense:** ACCCCCTGCTCTATGGCTTT	**Sn** 1215	105
		Antisense: GCTCTATGGCATCTGCATCCT	**As** 1319	
sst5TMD4	GQ359775	**Sense:** GTCCACCCTCTCCGCTCA	**Sn** 415	131
		Antisense: GCAGGTTCGCAGAGGACATC	**As** 545	
sst5TMD2	GQ359776	**Sense:** CAGTTCACCCGTACTGTGGCAT	**Sn** 358	132
		Antisense: CACAGCTTCAGGGTGGGTAA	**As** 489	
sst5TMD1	GQ359777	**Sense:** AACGTGTATATCCAGACAAGAGTGG	**Sn** 217	152
		Antisense: TCCCAGAAGACAACACCACA	**As** 368	
Mouse Pituitary hormones				
GH	NM_008117	**Sense:** CCTCAGCAGGATTTTCACCA	**Sn** 412	142
		Antisense: CTTGAGGATCTGCCCAACAC	**As** 553	
PRL	NM_011164.1	**Sense:** GGCCATCTTGGAGAAGTGTG	**Sn** 14	140
		Antisense: ACAGATTGGCAGAGGCTGAA	**As** 153	
POMC	NM_008895.3	**Sense:** GAGGCCTTTCCCCTAGAGTT	**Sn** 615	154
		Antisense: CACCGTAACGCTTGTCCTT	**As** 768	
TSH β-subunit	NM_009432.1	**Sense:** CTCCGTGCTTTTTGCTCTTG	**Sn** 177	156
		Antisense: TTGCCATTGATATCCCGTGT	**As** 332	
LH β-subunit	NM_008497.2	**Sense:** TGTCCTAGCATGGTCCGAGT	**Sn** 179	138
		Antisense: AGGAAAGGAGACTATGGGGTCTA	**As** 316	
FSH β-subunit	NM_008045.2	**Sense:** AGTTGATCCAGCTTTGCATCTT	**Sn** 70	245
		Antisense: GCCAGGCAATCTTACGGTCT	**As** 314	
Glycoprotein α-subunit	NM_009889.2	**Sense:** CTAGGAGCCCCCATCTACCA	**Sn** 242	168
		Antisense: CACTCTGGCATTTCCCATTAC	**As** 409	
Baboon Pituitary hormones				
GH	DQ340390	**Sense**: GACCTAGAGGAAGGCATCCAAA	**Sn** 21	143
		Antisense: AGCAGCCCGTAGTTCTTGAGTAG	**As** 163	
POMC	DQ315472	**Sense**: CCCTACAGGATGGAGCACTT	**Sn** 7	127
		Antisense: CGTTCTTGATGATGGCGTTT	**As** 133	
PRL	EF419886	**Sense**: CCTTCGAGACCTGTTTGACC	**Sn** 12	183
		Antisense: ATCTGTTGGGCTTGCTCCTT	**As** 194	
Ghrelin system				
Ghrelin	NM_021488.4	**Sense:** TCCAAGAAGCCACCAGCTAA	**Sn** 163	126
		Antisense: AACATCGAAGGGAGCATTGA	**As** 288	
In2-Ghrelin	DQ993169	**Sense:** GCTGTCTTCAGGCACCATCT	**Sn** 28	226
		Antisense: GTGGCTTCTTGGATTCCTTTC	**As** 253	
GHS-R	NM_177330.3	**Sense:** TCAGGGACCAGAACCACAAA	**Sn** 1002	71
		Antisense: CCAGCAGAGGATGAAAGCAA	**As** 1072	
GOAT	NM_001126314	**Sense:** ATTTGTGAAGGGAAGGTGGAG	**Sn** 473	120
		Antisense: CAGGAGAGCAGGGAAAAAGAG	**As** 592	

Supplemental table 1: Specific set of primers for genotyping of mouse cort-colony (cort+/+, cort+/- and cort-/-) by PCR of tail-snip DNA. Specific primers for amplification of mouse and baboon transcripts used for quantitative real-time RT-PCR.

Template	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
GH-sensitive liver genes				
IGF-I	NM_010512.3	**Sense:** TCGTCTTCACACCTCTTCTACCT	**Sn** 31	202
		Antisense: ACTCATCCACAATGCCTGTCT	**As** 232	
IGF-I var 1	NM_0105512.4	**Sense:** AGATCTGCCTCTGTGACTTCTTG	**Sn** 332	379
		Antisense: GATAGGGACGGGGACTTCTG	**As** 710	
IGF-I var 2	NM_184052.3	**Sense:** TCCTCTGGGATACGGCACTT	**Sn** 927	131
		Antisense: GAAGGTCTTGGTGGCATGTTT	**As** 1057	
IGF-I var 3	NM_001111274.1	**Sense:** CTACCAAAATGACCGCACCT	**Sn** 253	422
		Antisense: TGTACTTCCTTTCCTTCTCCTTTG	**As** 674	
IGF-I var 4	NM_001111275.1	**Sense:** GATCTGCCTCTGTGACTTCTTG	**Sn** 333	385
		Antisense: TGTGTTCTTCAAATGTACTTCCTTCT	**As** 717	
IGF-I var 5	NM_001111276.1	**Sense:** ACCTCGGCCTCATAGTACCC	**Sn** 203	433
		Antisense: TGTGTTCTTCAAATGTACTTCCTTCT	**As** 635	
IGF-ALS	NM_008340.3	**Sense:** GCTCAGCGTCTTTTGCAGTT	**Sn** 204	107
		Antisense: AGGGGATGGAGGACAGGTT	**As** 310	
MUP-3	NM_001039544.1	**Sense:** GAGCTTTTGTGGAAAACATCACT	**Sn** 263	104
		Antisense: TTGTTCACCAATCGCAGTCA	**As** 366	
GH-R	BC075720	**Sense:** GATTTTACCCCCAGTCCCAGTTC	**Sn** 1125	198
		Antisense: GACCCTTCAGTCTTCTCATCCACA	**As** 1322	
PRL-R	L14811	**Sense:** TGGGAGATCCACTTCACAGG	**Sn** 523	189
		Antisense: GGCCACAATGATCCACACA	**As** 711	
Hypothalamic-pituitary-related genes				
GHRH-R	NM_001003685.1	**Sense:** ACCCGTATCCTCTGCTTGCT	**Sn** 45	133
		Antisense: AGGTGTTGTTGGTCCCCTCT	**As** 177	
GHRH	NM_010285.2	**Sense:** TGCCATCTTCACCACCAAC	**Sn** 203	158
		Antisense: TCATCTGCTTGTCCTCTGTCC	**As** 360	
DR2	NM_010077.1	**Sense:** CGTTCTACGTGCCCTTCATC	**Sn** 719	139
		Antisense: TTGCCCTTGAGTGGTGTCTT	**As** 857	
PRLRH	NM_001101647.1	**Sense:** GCACCCCTGACATCAATCCT	**Sn** 95	154
		Antisense: GAGAACTTGGCACTTCCATCC	**As** 248	
PRLRH-R	NM_201615.2	**Sense:** TTACGTACGGGTGTCAGTGAAG	**Sn** 910	117
		Antisense: ACCACCACCAGCAGACAGAA	**As** 1026	
CRF	NM_205769.1	**Sense:** TCTGGATCTCACCTTCCACCT	**Sn** 630	95
		Antisense: CCATCAGTTTCCTGTTGCTGT	**As** 724	
CRF-R1	NM_007762.3	**Sense:** AACCTCATCTCGGCTTTCATC	**Sn** 659	193
		Antisense: ACGTGAGTACGATGGCTGTGT	**As** 851	
CRF-R2	NM_009953.2	**Sense:** CCACTGGAACCTCATCACCA	**Sn** 641	172
		Antisense: GTAGCAGCCCTCCACAAACA	**As** 812	
Ucn-2	NM_145077.1	**Sense:** GGTGTTCGTGGTCCTGATGT	**Sn** 33	115
		Antisense: TGAGGTCACAGAGCTAGGAGTTG	**As** 147	
Ucn-3	NM_031250.5	**Sense:** ATGCCCACCTACTTCCTGCT	**Sn** 522	149
		Antisense: GGCACATCTTCCAGCTTGTT	**As** 670	
NPY	NM_023456.2	**Sense:** GCTCTGCGACACTACATCAATC	**Sn** 224	107
		Antisense: GCGTTTTCTGTGCTTTCCTT	**As** 330	
GnRH	NM_008145.1	**Sense:** GCATTCTACTGCTGACTGTGTGTT	**Sn** 23	144
		Antisense: GTTCTGCCATTTGATCCACCT	**As** 166	
Kiss-1	NM_178260.3	**Sense:** AGCTGCTGCTTCTCCTCTGT	**Sn** 61	127
		Antisense: GCATACCGCGATTCCTTTT	**As** 187	
Gastrin	NM_010257.2	**Sense:** AAGATGCCTCGACTGTGTGTG	**Sn** 55	224
		Antisense: TTGGACAGGTCTGCTATGAAGTG	**As** 278	
TRH	NM_009426	**Sense:** CTGGCTTTGATCTTCGTGCT	**Sn** 137	157
		Antisense: GCACACGCTGGAGGTCTTT	**As** 293	
Mouse housekeeping genes				
Cyclophilin A	NM_008907.1	**Sense:** TGGTCTTTGGGAAGGTGAAAG	**Sn** 421	109
		Antisense: TGTCCACAGTCGGAAATGGT	**As** 529	
GAPDH	XM_001473623.1	**Sense:** ATGGCCTTCCGTGTTCCTAC	**Sn** 757	104
		Antisense: GCCTGCTTCACCACCTTCTT	**As** 860	
β-Actin	NM_007393.2	**Sense:** CTGGGACGACATGGAGAAGA	**Sn** 313	205
		Antisense: ACCAGAGGCATACAGGGACA	**As** 517	
HPRT	NM_013556	**Sense:** CAGTCAACGGGGGACATAAA	**Sn** 471	183
		Antisense: AGAGGTCCTTTTCACCAGCAA	**As** 653	
Baboon				
Cyclophilin A	DQ315437	**Sense:** CAAGACGGAGTGGTTGGATG	**Sn 351**	122
		Antisense: TGGTGGTCTTCTTGCTGGTC	**As 472**	

Supplemental Table 2: Absolute mRNA copy numbers [adjusted by normalization factor calculated from the mRNA copy number of 2-4 separate housekeeping genes (see material and method section)] of hypothalamic, stomach and liver transcripts of male and female cort +/+ and cort -/- mice obtained by quantitative real-time RT-PCR method. Values represented the means ± SEM of n=5-8 mice/genotype/gender.

	Males				Females			
	cort +/+		cort -/-		cort +/+		cort -/-	
HYPOTHALAMIC	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
GHRH	13526,5	1312,8	14543,9	822,6	10109,5	615,7	11404,0	416,4
SST	171167,9	8515,8	170008,6	17319,4	141988,5	7920,3	134002,5	19468,8
sst1	20483,1	2034,2	24134,8	3723,6	23711,2	1155,1	20649,7	2065,4
sst2A	21718,2	1310,4	23839,3	2111,6	21085,7	1001,6	19040,6	737,5
sst2B	4952,4	275,5	5417,2	232,8	4871,3	445,1	4135,0	168,8
sst3	16914,6	1052,6	16318,4	758,8	16593,9	1291,7	16223,6	646,4
sst4	19668,3	2336,2	22113,9	2884,8	18676,7	1492,1	18300,5	1661,9
sst5	16239,8	1437,5	16231,0	1140,0	15830,1	1030,3	14159,3	993,9
sst5TMD4	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
sst5TMD2	11870,4	1178,9	11917,9	1109,7	13126,2	1075,0	11090,1	955,8
sst5TMD1	14,6	1,6	19,8	2,4	21,8	4,3	14,0	1,3
Ghrelin	18,1	1,4	16,2	1,4	13,0	1,9	16,9	2,4
In2-Ghrelin	8102,6	713,5	7093,0	469,5	7758,3	1234,5	6889,2	726,9
GHS-R	15666,3	1463,2	18001,5	1214,6	17086,7	2156,4	12685,8	1411,0
GOAT	86,4	6,9	82,7	1,6	86,6	13,3	73,9	4,7
PRLRH	269,4	33,2	270,9	30,7	379,8	48,9	401,3	59,3
CRF	4404,9	462,8	4556,4	611,9	5682,6	524,7	5058,2	615,5
Ucn2	15,7	2,2	21,1	4,2	25,7	8,3	11,4	1,0
Ucn3	269,4	33,2	270,9	30,7	379,8	48,9	401,3	59,3
POMC	49595,1	12090,4	60328,1	12134,6	40709,6	2242,5	42697,4	3692,7
NPY	182076,2	14022,7	199766,4	7116,3	166595,5	33719,0	141133,7	7065,3
TRH	37195,6	10968,7	42288,2	5786,3	35084,6	8051,7	35809,7	2007,8
GnRH	1437,5	224,0	1846,2	578,3	2003,1	451,6	1790,9	167,2
Kiss1	1366,6	95,4	1499,7	103,5	4396,6	893,8	4732,2	444,7

	Males				Females			
	cort +/+		cort -/-		cort +/+		cort -/-	
STOMACH	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Gastrin	87679,1	12788,0	78962,2	20192,6	80822,6	25316,6	66628,2	12275,7

	Males				Females			
	cort +/+		cort -/-		cort +/+		cort -/-	
LIVER	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
IGF-I	161958,1	13371,9	179215,9	9370,4	182185,9	13472,0	177888,5	14687,2
IGF-I ALS	11894,2	1151,3	14766,6	3299,1	25754,8	4261,7	16387,1	2007,5
IGF-I var1	39429,2	5737,1	45151,1	9026,9	35799,4	4950,5	36289,6	5620,6
IGF-I var2	3308,9	1579,8	1217,9	328,9	1271,4	370,5	908,4	195,5
IGF-I var3	14912,5	1239,4	14355,9	1685,5	14100,1	1328,0	14369,9	1373,0
IGF-I var4	73254,3	5885,9	83681,2	6947,8	85439,4	6495,6	81920,6	10955,5
IGF-I var5	8086,9	428,4	7313,1	1017,6	7214,9	1077,1	7958,2	858,7
GH-R	55340,8	2216,2	48851,5	3176,1	52824,3	5394,2	41183,6	5001,0
PRL-R	43250,7	10175,7	46061,3	3040,2	196965,4	21949,2	176393,2	27353,2
Mup-3	5092503,2	438654,6	4910509,8	584419,9	1560094,0	139273,0	1488227,5	187865,0

Article IV

Low doses of somatostatin signal through sst5 and AC/cAMP to dramatically increase GH release in primary pituitary cell cultures from a non-human primate (Papio anubis).

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Abstract

Somatostatin (SST) and cortistatin (CST) are classical inhibitors of growth hormone (GH) release. However, earlier results in porcine somatotropes suggested that, al low doses, they may also stimulate GH secretion. In this report primary pituitary cell cultures from normal adult female baboons (non-human primate model) were used to study the impact of SST and CST treatment on GH release. High doses (10^{-10} M or greater) of SST (and CST) had no effect on basal GH secretion but blocked GH-releasing hormone (GHRH)- and ghrelin-induced GH release. In contrast, a wide range of low doses of SST (and CST; 10^{-17} to 10^{-13} M) dramatically stimulated GH release to levels comparable to those evoked by GHRH and ghrelin. Using SST-receptors (sst) specific agonists and antagonists, along with pharmacological intracellular signal transduction blockers, the inhibitory actions of high-dose SST were attributed to sst2 and sst1 activation and required an intact AC and MAPK system. In contrast, the stimulatory actions of low-dose SST were attributed to sst5 signaling through AC/cAMP/PKA and intracellular Ca2+, where both, low-dose SST or sst5-agonists, augmented ghrelin-induced, but not GHRH-induced GH release. Importantly, both low-dose SST and sst5-agonists inhibited prolactin release. Taken together, these results unveil an important stimulatory action of SST and CST on primate GH release and demonstrate that the ultimate impact of SST/CST on pituitary hormone release from primate pituitary cells is dose-dependent, as well as, cell type- and receptor-specific.

Introduction

It is commonly accepted that somatostatin (SST) acts as the primary physiological inhibitor of somatotrope function by blocking basal and/or growth hormone-releasing hormone (GHRH)- and ghrelin-stimulated GH secretion [1, 2]. However, our group and others have demonstrated that SST can function as a true GH-releasing factor in pig primary pituitary cell cultures when delivered at a low dose (subfemtomolar to fentomolar range) [3-8]. Of note, cortistatin (CST), a peptide structurally similar to SST, also binds and activates SST receptors (sst), and can also stimulate GH release in primary cultures prepared from pigs [6] and rats [9]. In the pig, these divergent effects have been attributed to SST activation of distinct receptors, where the inhibitory effect of SST and CST is mediated primarily by activation of the SST receptor subtypes, sst1 and sst2, while the stimulatory effect of SST is mediated by sst5 signaling through AC/cAMP/PKA [5, 10]. Also, in primary pituitary cell cultures from chickens, Bossis and Porter [11] used the reverse hemolytic plaque assay, which measures the relative amount of hormone released from single cells, and showed that although a sst5 agonist reduced the proportion of somatotrope secreting low levels of GH, it increased the proportion of somatotropes releasing high levels of GH, suggesting a subset of somatotropes are sensitive to the stimulatory effect of sst5 activation as, it has been previously shown in a subpopulation (high-density) of porcine somatotropes [5, 10]. It is also possible that sst5 could stimulate GH release in human somatotropes, since cell lines (CHO-K1 and COS-7) stably transfected to express the human sst5, respond to SST treatment with an increase in intracellular cAMP accumulation [12, 13], which can be blocked by a dominant negative Gαs (Gαs acetyl 354–372) [13], where Gαs-mediated cAMP induction is required for GH release [14, 15]. This may explain in part reports showing patients with GH-producing pituitary adenomas that express higher levels of sst5, tend to show a poorer response to SST agonist therapy [16, 17]. It has also been reported, but not emphasized in the literature, that a small subset of human GH-producing pituitary adenomas release GH *in vitro* in response to SST or SST receptor agonist challenge [18-22]. However it should be noted the paradoxical responses observed in adenomas are not always clearly associated with sst5 expression.

Although the data collected thus far on the stimulatory actions of SST are intriguing, it remains controversial and may be limited to particular species or patho-physiologic conditions. Therefore in the current study, primary pituitary cell cultures from normal female baboons (*Papio anubis*), a species that over the last years have been turned as a non-human primate model to study different levels of human physiology (including pituitary function) [23-28] were used to test the impact SST and sst subtype agonists on basal and GHRH- and ghrelin-stimulated

GH release, using a wide range of doses. In addition, sst-subtype antagonists, as well as, intracellular signal transduction blockers were used to define the receptor/intracellular signaling pathways critical for both the stimulatory and inhibitory action of SST on primate GH release.

Methods

Culture reagents

SST-14, CST-17, GHRH and ghrelin were purchased from Sigma and Phoenix Pharmaceuticals (Burlingame, CA). Minimum essential media (α-MEM), HEPES, horse serum and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY). Inhibitors of intracellular signaling pathways were purchased from Sigma-Aldrich (MDL-12,330A, H-89, thapsigargin, nifedipine, GO6983 and PD-98,059), Cayman Chemical (U73122, LY-83,583 and L-NAME; Ann Arbor, MI). Subtype selective agonists for sst1 (L-797,591 and BIM-23926), sst2 (L-779,976 and BIM-23120), sst3 (L-796,778), sst4 (L-803,087), sst5 (L-817,818 and BIM-23206) and antagonists for sst-2 (BIM-23627), sst5 (BIM-23867) and GHS-R1a (BIM-28163) were generously provided by Merck & CO., INC. (Whitehouse Station, NJ) [29] or IPSEN Biomeasure (BIM compounds; Milford, MA) [30, 31]. All other reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Animals and pituitary collection

Pituitaries were obtained from random cycling female baboons (*Papio anubis*, 7-20 years of age). These animals represent control animals from studies conducted by other University of Illinois at Chicago investigators, where all studies were approved by the Institutional Animal Care and Use Committee. Within 15 minutes of euthanasia (sodium pentobarbital overdose), pituitaries were immediately excised and placed in sterile cold (4C) basic media consisting of α-MEM, 0.15% bovine serum albumin, 6 mM HEPES, and 10 IU/ml penicillin and 10 µg/ml streptomycin. Pituitaries were washed twice in fresh media and cut into smaller fragments with surgical blades. Some fragments were rapidly frozen in liquid nitrogen and stored at -80C until RNA isolation (see below for details), while the remaining fragments were dispersed into single cells for culture as described below.

Primary pituitary cell culture

Anterior pituitaries were dispersed into single cells by enzymatic and mechanical disruption, as previously described [25, 27, 32]. Cells were plated onto 24-well tissue culture plates at 200,000 cells/well in 0.5 ml of basic medium containing 10% horse serum. After a 48h-incubation (37C), medium was removed and cells were pre-incubated for 1h in fresh, warm (37C) serum-free medium to stabilize basal hormone secretion. Following the pre-incubation period, medium was replaced with serum-free medium containing treatments as described below.

Experiment 1: **A)** In order to determine if SST or CST had dose dependent effects on GH release, cultures were incubated with SST or CST alone for 4h (from 10^{-19} to 10^{-7} M) or 24h (10^{-15} and 10^{-7} M); **B)** To investigate if SST or CST could alter GHRH-, ghrelin- or low dose CST-stimulated GH-release, culture were incubated with SST alone (10^{-15} or 10^{-7} M) or in combination with GHRH, ghrelin (10^{-8} M) or CST (10^{-15} M) for 4h.The dose of GHRH and ghrelin were selected according to previous studies [25].

Experiment 2: **A)** In order to determine whether different sst-subtypes mediate the inhibitory and stimulatory actions of SST/CST on GH release, we used selective agonists for all sst-subtypes alone or in combination with GHRH or ghrelin for 4h. **B)** In addition, we employed antagonists for sst2 and sst5 to determine whether the actions observed with agonists for these receptors were specific.

Experiment 3: In order to study the intracellular signaling pathways involved in the actions of SST (as well as of sst2 and sst5 agonists) on pituitary function, medium containing the inhibitors of key intracellular signaling pathways were added following the 1h pre-incubation period (medium alone was used in the vehicle-treated controls). Ninety minutes later, the medium was replaced with medium alone (vehicle-treated controls) or containing the selected inhibitor combined with SST (10^{-15}M), sst2-agonist (10^{-7}M) or sst5-agonist (10^{-11}M) and incubated for an additional 4h period.

In all experiments, medium was collected for hormone analysis (see below). Controls consisted of cells cultured in serum-free basic medium. Each treatment was repeated at least 3 times on different pituitary cell preparations (3-4 wells/treatment/experiment).

Hormone and cAMP analysis

Culture media was recovered, centrifuged (2000g/5 min) and stored at –80C for subsequent analysis of GH concentrations

using a commercial hGH ELISA kit (DSL, Webster, Texas or DRG, Mountainside, NJ). In addition, to verify the specificity of the response, prolactin (PRL) levels were measured on select samples using a hPRL ELISA (DSL). In some cultures, media were removed, lysis buffer was added, and cell lysates were recovered and stored at –80 C for analysis of intracellular cAMP accumulation, as assessed by the cAMP Biotrack EIA system following protocol 3 of the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

RNA isolation, reverse transcription (RT) and quantitative real-time PCR (qrtRT-PCR)

Total RNA from primary pituitary cell cultures and whole pituitary tissue was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) with deoxyribonuclease treatment, as previously described [25, 27, 32]. The amount of RNA recovered was determined by the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA was reverse-transcribed in a 20µl volume using random hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). The cDNA obtained was treated with Ribonuclease H (1U, MRI Fermentas) and duplicate aliquots (1µl) were amplified by quantitative real-time RT-PCR (qrtRT-PCR) using the Stratagene Brilliant SYBR green QPCR Master Mix. Details regarding the development, validation and application of qrtRT-PCR to measure expression levels of different baboon transcripts, including *cyclophilin-A* (used as a housekeeping gene), have been recently reported by our laboratory [25-27, 32]. New baboon sequences obtained in the present study (sst1-sst5) were submitted to GenBank. Primer sets for baboon *sst1-sst5, GHRH-R, GHS-R, GH, PRL and cyclophilin-A* used in this study, as well as the GenBank accession numbers, are provided in Supplemental Table1.

Statistical analysis

In order to normalize mRNA values within each treatment and minimize intra-group variations, the values obtained were compared to vehicle-treated controls (set at 100%) and the results are reported as the mean ± SEM in all experiments. Each treatment group was tested on a minimum of 3 separate pituitary cultures each prepared from a different animal, and within each pituitary cell preparation (experiment) treatments were replicated in at least 3-4 wells. Data were assessed for heterogeneity of variance, and if found, values were log-transformed. Differences between treatment groups were assessed by analysis of variance (1-way or 2-way ANOVA) with repeated measures, followed by Fisher's test for multiple comparisons. $p<0.05$ was considered significant. All statistical analyses were performed using GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD).

Results

Baboon primary pituitary cell cultures as an appropriate model to predict how SST modulates human somatotrope function

Pituitaries from female baboons expressed all sst-subtypes, however, the relative expression levels varied between sst-subtype with sst5>sst2>sst1>sst3>sst4 (table-1). These results are closely similar to the expression profile showed in normal human pituitaries [33]. Comparison of partial baboon mRNA sequences of target genes [generated in this and previous studies [25, 27, 32] with the corresponding human sequences revealed a close homology [*sst1 98%, sst2 98%, sst3 97%, sst4 96%, sst5 95%, GH 98%, PRL 97%, SST 98% and CST 95%*), with a higher degree of divergence vs. gene sequences from non-primate species (78-95% homology when compared with transcripts of pig, rat, mouse, cattle, dog and sheep). To confirm that primary pituitary cells of baboons maintain a differentiated phenotype after dispersion and culture, absolute mRNA levels (copy numbers/0.05 µg total RNA) of all sst-subtypes, GHRH-R, GHS-R, GH, PRL and *cyclophilin A* were compared between whole tissue extracts and extracts prepared from pituitary

Table1: Absolute cDNA copy number/0.05µg total RNA of gene transcripts in the whole pituitary versus primary pituitary cell cultures (control groups) of female baboons, as determined by quantitative real-time RT-PCR.

	Whole pituitary	Primary pituitary cell cultures
sst1	899 ± 118	885 ± 104
sst2	2,182 ± 229	2,018 ± 309
sst3	212 ± 23	198 ± 27
sst4	34 ± 5	29 ± 4
sst5	4,465 ± 357	3,809 ± 479
GHRH-R	4,731 ± 428	4,441 ± 813
GHS-R	1,123 ± 141	1,302 ± 177
GH	291,475 ± 29,302	262,077 ± 35,588
PRL	1,143,067 ± 73,030	1,075,048 ± 134,167
Cyclophilin A	79,469 ± 5,578	88,280 ± 1,626

Values represent means ± SEM (from 3 separate whole pituitary extracts and primary pituitary cell cultures of the same baboons)

cultures 4h after incubation in serum-free media (table-1). Transcript levels did not vary significantly between *in vivo* and *in vitro* samples, indicating that the cell preparation and culture conditions did not have an adverse effect on the expression of transcripts important in this study. It should also be noted that PRL expression levels were greater than GH in pituitary tissue extracts and cell culture preparations from these female baboons, consistent with previous reports demonstrating that lactotropes are more abundant than somatotropes in female pituitaries [25, 34]. Taken together, these results indicate that the culture system used allows for the maintenance of correct pituitary cell function and suggests that information gained in this study could model the effects of SST/CST in human pituitaries.

Direct effect of SST and CST on pituitary hormone release

Both SST and CST stimulated basal GH release at low concentrations ranging between 10^{-13} to 10^{-17} M, whereas high concentrations (10^{-10} and 10^{-7} M) did not affect basal GH secretion (Fig-1A, right and left panels, respectively). Based on the above results, we selected a low dose of 10^{-15} M (lowest dose that caused maximal GH secretion) and a high dose of 10^{-7} M to further analyze the action of these peptides. Combined administration of low doses of SST and CST increased GH release to the same level as that observed when each peptide was applied separately (Fig-1B), suggesting that SST and CST work through similar intracellular signaling pathways to release GH. Given the limited source of baboon pituitary tissue, subsequent studies were performed focusing on the effect of SST. We found that the stimulatory effect of low doses of SST on GH release was still evident at 24h of incubation (Fig-1C), which was also reflected by a significant increase in GH mRNA (data not shown). Interestingly, the effect of SST on pituitary GH release was specific in that both low and high doses of the peptide significantly inhibited PRL release (4h-incubation; Fig-1D).

Interaction of SST with major regulators of GH (GHRH and Ghrelin) release

As shown in Fig-1E (left-panel), a high dose (10^{-7} M) of SST did not alter basal GH release but completely blocked GHRH- and ghrelin-stimulated GH secretion. Interestingly, the stimulatory effect of low-dose SST (10^{-15} M) on GH release was similar to that of GHRH or ghrelin, delivered at a dose previously demonstrated to evoke maximal GH release [25] (Fig-1E, right-panel). Interestingly, co-administration of low-dose SST with ghrelin, but not with GHRH, elicited an additive increase on GH release, as compared to the effects of each peptide alone (Fig-1E, right-panel), suggesting that low-dose SST and GHRH may share common intracellular signaling pathways, which are distinct from ghrelin.

Intracellular signaling pathways required for low-dose SST-mediated GH release

Low doses SST or CST increased intracellular cAMP accumulation (Fig-2A). In contrast, high doses of SST and CST did not affect basal intracellular cAMP levels (Fig-2A), consistent with the lack of effect on GH release (Fig-1A). Treatment of pituitary cell cultures with specific inhibitors of AC, PKA, extracellular Ca^{2+} influx or NOS, but not with blockers of PLC, PKC, intracellular Ca^{2+} influx, GC and MAPK, completely suppressed the stimulatory effects caused by low-dose SST on baboon GH release (Fig-2B). Administration of these inhibitors alone did not modify basal GH secretion (Fig-2B).

Effect of selective agonists for all sst-subtypes on somatotrope function

Owing to the limited availability of baboon cell preparations and based on the results presented in figure-1, we initially selected a high (10^{-7} M) and a low (10^{-15} M) dose of sst1-5 agonists (Merck) to test their effect on basal GH release. As shown in Fig-3A, agonists selective for sst1, sst3 and sst4 did not alter basal GH release at low or high doses, while a high dose of sst2-agonist inhibited GH secretion. In contrast, the sst5 agonist increased GH release at doses ranging from 10^{-15} to 10^{-9} M (Fig-3B), where the maximal effect was observed at 10^{-11} M. Therefore, 10^{-11}M was chosen to further analyze the action of the sst5-agonist. Interestingly, we found that high (10^{-7} M) and low (10^{-11} M) doses of sst5-agonist significantly inhibited PRL release, while sst1 and sst2-agonist did not alter PRL secretion (Fig-3C).

We next investigated the possible interaction of sst1-5 agonists with GHRH or ghrelin. As illustrated in figure-3D, high dose of sst1 or sst2 (10^{-7} M), but not sst3 or sst4, agonists blocked GHRH- and ghrelin-stimulated GH release. High or low doses of the sst5-agonist did not modify GHRH-stimulated GH release (Fig-3E). Interestingly, a high dose of the sst5-agonist

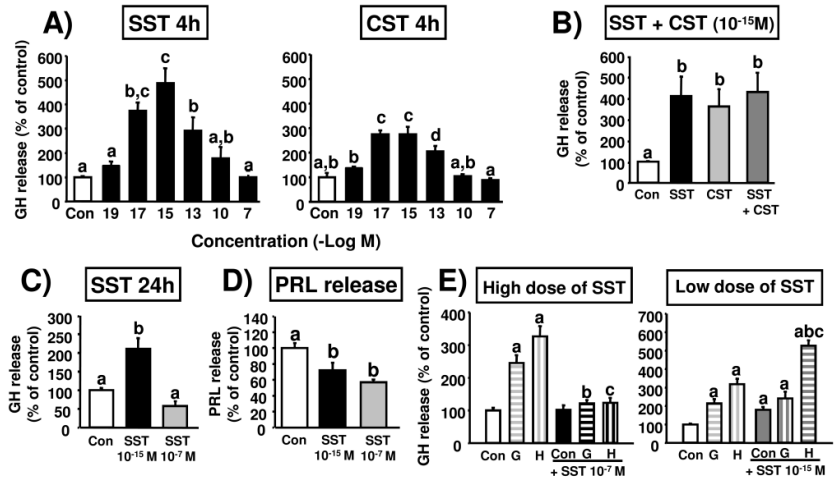

Figure 1: Direct effect of somatostatin (SST) and cortistatin (CST) on GH and PRL release and interaction with GHRH and ghrelin. (**A)** Effect of SST and CST treatment (4h) on GH release from baboon pituitary cell cultures. **(B)** Interaction of low doses (10^{-15} M) of SST and CST on GH secretion. **(C)** Effect of 24h treatment of SST at low dose (10^{-15} M) and high dose (10^{-7} M) on baboon GH release. **(D)** Effect of 4h treatment of SST at low dose (10^{-15} M) and high dose (10^{-7} M) on baboon PRL release. **(E)** Interaction of high dose (10^{-7} M; left panel) or low dose (10^{-15} M; right panel) of SST with GHRH or ghrelin (10^{-8} M) on baboon GH secretion. For figures 1 (A), (B), (C) and (D): values that do not share a common letter (a, b, c or d) significantly differ ($P < 0.05$). For figure 1(E), $P < 0.05$: a, vs. control; b, vs. GHRH alone; and c, vs. ghrelin alone. Data are expressed as percentage of controls (Con), set at 100% within each experiment, and represent the mean ± SEM of 3-7 independent experiments (3-4 wells/treatment/expt).

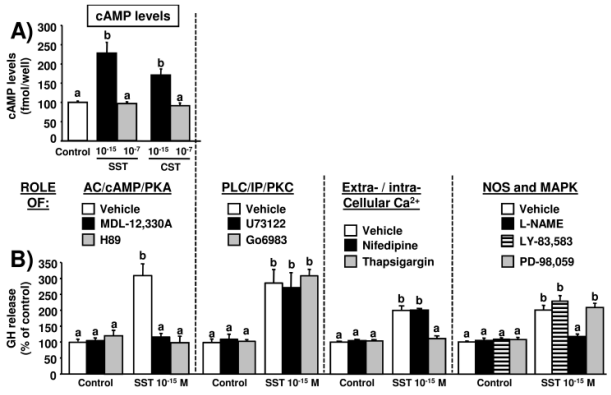

Figure 2: Intracellular signaling pathways of low dose of somatostatin-stimulated baboon GH release. (A) Effect of low (10^{-15} M) and high (10^{-7} M) doses of somatostatin and cortistatin on cAMP accumulation in primary pituitary cell cultures from baboon. **(B)** Effect of inhibition of AC (MDL-12,330A; 10 µM), PKA (H89; 15 µM), PLC (U73122; 50 µM), PKC (Go6983; 20 µM), extracellular Ca^{2+} channels (nifedipine; 1 µM), intracellular Ca^{2+} channels (thapsigargin; 10 µM), NOS (L-NAME; 10 µM), GC (LY-83,583; 10 µM), and MAPK (PD-98,059; 10 µM) on low dose of somatostatin-stimulated baboon GH release. On the day of the experiment, inhibitors were added to the incubation media 90min before low dose of somatostatin treatment (4 h; 10^{-15} M) Values are expressed as percentage of vehicle-treated controls without inhibitor (set at 100%) within each experiment, and represent the mean ± SEM of 3-5 independent experiments (3-4 wells/treatment/expt). Values that do not share a common letter (a or b) significantly differ ($P < 0.05$).

blunted, but did not eliminate, ghrelin-stimulated GH release; while a low dose, combined with ghrelin, had an additive effect (Fig-3E).

We also studied intracellular cAMP accumulation levels in response to sst1, sst2 and sst5 agonist alone or combined with ghrelin (Fig-3F). Specifically, we observed that a high dose of sst2-agonist inhibited, while only a low dose of sst5-agonist increased, cAMP levels. This was consistent with the effects of these agonists on GH release (Fig-3A and B). Moreover, despite ghrelin's inability to enhance cAMP levels, when applied alone, ghrelin did augment the stimulatory action of low dose sst5-agonist (Fig-3F).

Use of pharmacologic blockade of intracellular signals suggests that the stimulatory action of the sst5 agonist is mediated through the same signaling pathway as low-dose SST (AC/PKA; Fig-3G). We also studied selected signaling routes involved in sst2 agonist-inhibition of GH release and observed that an intact MAPK system, as well an intact AC/PKA system, is required for sst2-mediated inhibition of GH release (Fig. 3G).

Effect of selective antagonist for sst2 and sst5 on sst2- and sst5-induced GH release

Use of alternative sst-agonists (Ipsen) confirmed previous results showing the sst5-agonist BIM-23206, stimulated (doses ranging from 10^{-7} to 10^{-13} M; Fig-4A and 4B), while the sst2-agonist, BIM-23120, inhibited (10^{-7} M; Fig-4C) GH release. Of note, we observed that a specific sst5-antagonist, BIM-23867, completely blocked GH release in response to low doses of SST (10^{-15} M) and sst5-agonists (L-817,818 and BIM-23206; 10^{-11} M) (Fig-4B), while an sst2-antagonist blocked the inhibitory effect of sst2 agonists (L-779,976 and BIM-23120; 10^{-11} M) on basal GH secretion (Fig-4C). The additive effect on GH release observed with the co-administration of ghrelin and low dose of SST was blunted in the presence of the sst5- or GHS-R-antagonist, but completely blocked by the presence of both antagonists (Fig-4D), confirming the additive effect of sst5 agonists and ghrelin requires both sst5 and GHS-R.

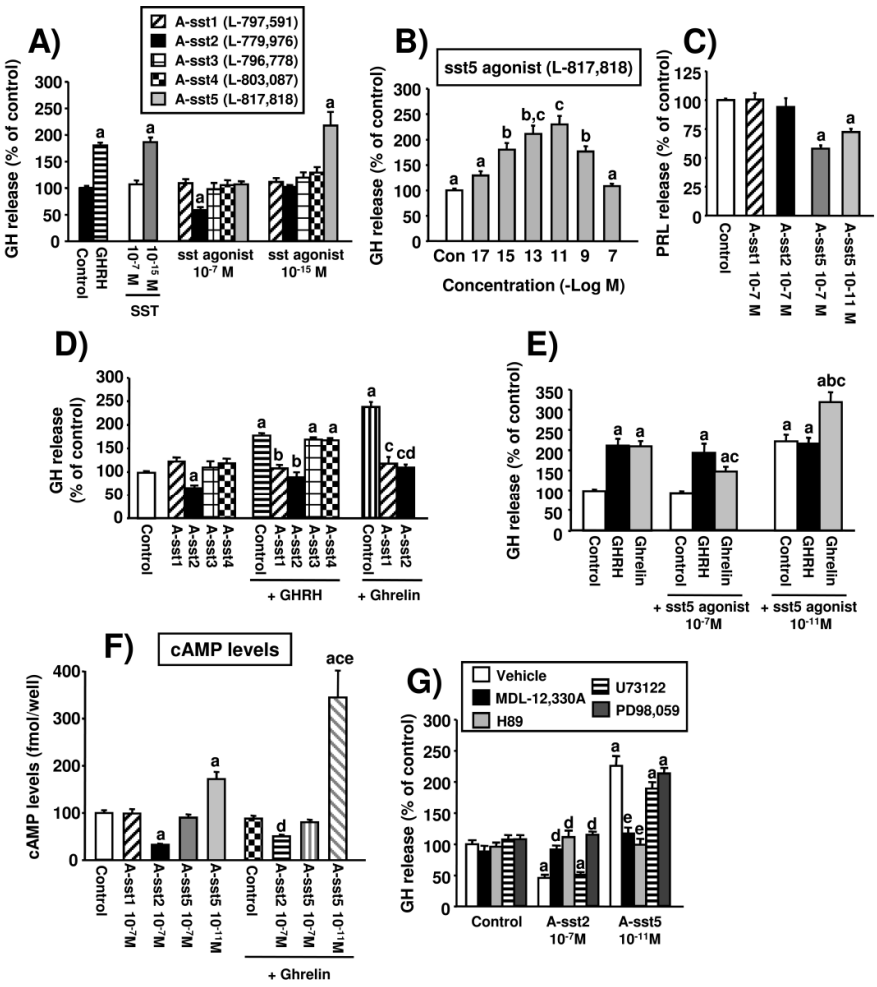

Figure 3: Direct effect (4h) of sst1-5 agonists on GH and PRL release and their interaction with GHRH and ghrelin. **(A)** Effect of sst1-5 agonists treatment (10^{-15} M and 10^{-7} M) on GH release from baboon pituitary cell cultures. **(B)** Dose response effect of sst5 agonist (10^{-17} to 10^{-7} M) on GH release. **(C)** Effect of 4h treatment of agonists of sst1 (10^{-7} M), sst2 (10^{-7} M) and sst5 (10^{-7} M and 10^{-11} M) on baboon PRL release. **(D)** Interaction of sst1-4 agonists (10^{-7} M) with GHRH and/or ghrelin (10^{-8} M) on baboon GH secretion. **(E)** Interaction of high (10^{-7} M) or low (10^{-11} M) dose of sst5-agonist with GHRH and ghrelin (10^{-8} M) on baboon GH secretion. **(F)** Effect of agonists of sst1 (10^{-7} M), sst2 (10^{-7} M) and sst5 (10^{-7} M and 10^{-11} M) alone or in combination with ghrelin (10^{-8} M) on cAMP accumulation in primary pituitary cell cultures from baboon. **(G)** Effect of inhibition of AC, PKA, PLC or MAPK on sst2 agonist-inhibited (10^{-7} M) and sst5 agonist-stimulated (10^{-11} M) baboon GH release. For figures 3 A, C-G: $P < 0.05$: a, vs. control; b, vs. GHRH alone; c, vs. ghrelin alone; d, vs. 10^{-7} M sst2-agonist alone; and e, vs. 10^{-11} M sst5-agonist alone. For figure 3 (B): values that do not share a common letter (a, b or c) significantly differ ($P < 0.05$). Data are expressed as percentage of controls (Con), set at 100% within each experiment, and represent the mean ± SEM of 3-5 independent experiments (3-4 wells/treatment/expt).

Discussion

Our understanding of the direct pituitary effects of SST on GH secretion has been largely formed by studies conducted in non-primate species and pituitary cell cultures established from human fetal pituitaries or GH-producing adenomas [2, 19, 22, 35-45]. For the most part, the results indicate that SST suppresses basal and/or GHRH- and ghrelin-stimulated GH release, depending on the model studied and the dose tested [2, 35, 38, 39, 46]. To date an appropriate model system has not been used to predict how SST directly modulates GH release in normal humans and the intracellular signaling pathways activated by SST or SST-analogs to exert these actions. Therefore, in this report we used primary pituitary cell cultures from normal adult female baboons (non-human primate model) to study the impact of SST and CST treatment on GH release. We observed that high doses (10^{-10} M or greater) of SST (and CST) had no effect on basal GH secretion but blocked GHRH- and ghrelin-induced GH release. In contrast, our results clearly demonstrate that a wide range of low doses of SST (and CST; 10^{-17} to 10^{-13} M) can actually stimulate GH release, consistent with that previously reported in primary pituitary cell cultures from pigs [3-8, 10].

In this study, all five SST receptors were expressed in whole pituitary extracts and pituitary cell cultures from baboons, where the relative pattern of expression (sst5>sst2>sst1>sst3>sst4) was similar to that observed in normal human pituitaries [33]. Using in situ hybridization [47, 48], immunocytochemistry [49] and mRNA expression profiles of purified somatotrope populations ([33, 50-52], sst2 and sst5 have been shown to be the most dominant subtypes in somatotropes, although sst1 and sst3 are also expressed. However in the current study, only the sst2 and

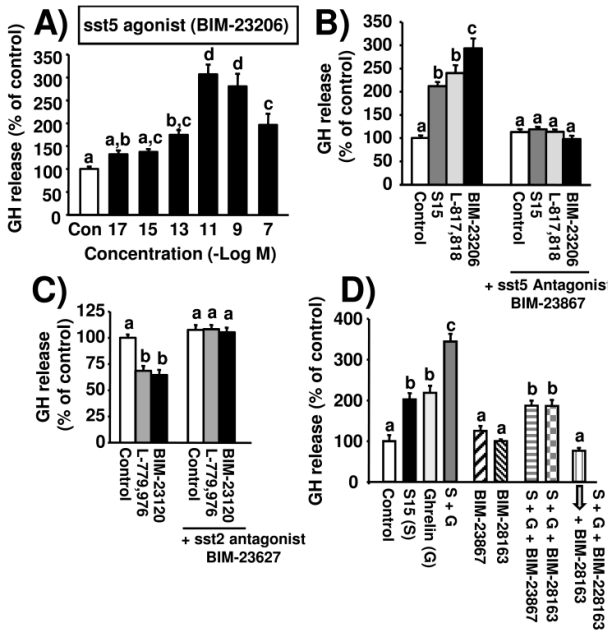

Figure 4: Direct effect sst2 and sst5 antagonist (4h) on GH release. A) Dose response effect of sst5 agonist (BIM-23206; 10^{-17} to 10^{-7} M) on GH release. **B)** Effect of antagonist of sst5 (BIM-23867) on low dose somatostatin-stimulated (10-15 M) and sst5 agonist (L-817,818 and BIM-23206)-stimulated (10^{-11} M) GH release. **C)** Effect of antagonist of sst2 (10^{-8} M) on sst2 agonist-inhibited (10^{-7} M) GH release. **D)** Effect of antagonists of sst5 (BIM-23867) and/or ghrelin receptor type-1a (BIM-28163; 10nM) on combination of low dose somatostatin and ghrelin-stimulated GH release. Values are expressed as percentage of vehicle-treated controls (set at 100%) within each experiment, and represent the mean ± SEM of 3-7 independent experiments (3-4 wells/treatment/expt). Values that do not share a common letter (a, b, c or d) significantly differ ($P < 0.05$).

sst1 agonists could block GHRH and ghrelin-stimulated GH release, while the sst2 agonist also reduced basal GH output. These results strongly suggest that high-dose SST inhibits GHRH/ghrelin-stimulated GH release by activation of sst2 and sst1. The fact that sst2 agonists, and not high dose SST, could block basal GH release may be related to the fact that SST binds to all sst subtypes, where each is known to differentially couple to G-proteins [39] and upon ligand binding can form homo- or hetero-dimers that can in turn modify their association with intracellular signals [53-58]. In support of our current results, studies using SST receptor specific agonists/antagonists in GH-producing cell lines, primary pituitary cultures (pigs, chickens, rats) and/or human pituitary GH-producing adenomas cultures have revealed only sst2 consistently confers the inhibitory actions of SST on GH release, while activation of the other SST receptor subtypes are variable depending on the dose of agonist supplied and the model tested [5, 11, 35, 46, 54, 59-68]. It has also be shown, using non-primate models that sst2-mediated inhibition of GH release requires intact AC activity and is associated with inhibition of cAMP accumulation and voltage gated Ca^{2+} channels, and/or stimulation of membrane K^{+} channels [2, 5, 11, 35, 54, 59-64, 69]. The current study show for the first time in a primate species model that sst2 can inhibit basal cAMP accumulation and requires an intact AC/PKA system to mediate its inhibitory effects on GH release. Interestingly, we also observed that intact MAPK signaling is required for the inhibitory action of the sst2 agonist on basal GH release. We have previously reported that ghrelin-, but not GHRH-stimulated GH release requires MAPK in baboon primary pituitary cell cultures [25]. It has also been reported that SST and a SST agonist (octreotide) can inhibit MAPK activity, where this effect has been associated with the ability of SST to inhibit proliferation and induce apoptosis in pituitary cell cultures [35, 70, 71]. However, to our knowledge, this is the first report showing a link between SST/sst2/MAPK and inhibition of basal GH release. Although caution must be taken in interpreting results derived from the use of pharmacological blockers to study the inhibitory actions of ligand/receptor activation, these results are intriguing and set the stage for future investigations.

In addition to confirming the uncontested inhibitory actions of high-dose SST on GH release, the current results demonstrate low-dose SST and CST can stimulate GH release in primate pituitary cell cultures, consistent with that observed in cultures from normal pigs and rats [3, 4, 6-10], and in a select population of human pituitary adenomas [18-22]. In fact, in the baboon model, the magnitude of GH release evoked by low-dose SST was similar to that observed for maximal doses of GHRH and ghrelin. Therefore, the paradoxical stimulatory effects of low-dose SST/CST extend across species and may in fact be important in regulation of normal somatotrope function in humans. Results obtained using sst subtype-specific agonists/antagonists and intracellular signal transduction blockers strongly suggest that the stimulatory effects of low-dose SST are mediated through sst5 activation of AC/cAMP/PKA, intracellular calcium influx, and NOS, the same network of intracellular pathways that are critical for GHRH-induced GH release [15, 25, 72].

It should be emphasized that the stimulatory actions of SST and sst5 agonists on hormone release was not ubiquitous, since both low-dose SST and sst5 agonists inhibited PRL release. This is consistent with the observation that sst5 selective agonists were as effective as carbergoline in suppressing PRL release from rat pituitary cells [73] and human prolactinomas [44, 74]. These results, taken together with our current findings indicate that the ultimate impact of ligand–mediated sst5 signaling on

hormone release is cell-type specific. Not only can the actions of sst5 differ between cell types, it has also been reported that the actions of sst5 can be modulated by the presence of other G-protein coupled receptor types. Specifically, Zatelli et al [75], observed that somatotropinomas that express sst2, sst5 and DR2, are insensitive to sst5 agonist inhibition of GH release, where in fact some adenomas showed a positive response. In contrast, the sst5 agonist inhibited GH release in somatotropinomas that expressed sst2 and sst5, but lacked DR2.

Although low-dose SST or sst5 agonists did not alter GHRH-induced GH release, combined treatment with ghrelin did result in an additive effect on GH release, where both sst5 and GHS-R are required, as demonstrated by the use of receptor-specific antagonists. This additive effect may be due to the activation of distinct intracellular signal transduction pathways, as it has been previously shown that ghrelin requires PLC/PKC, MAPK, and extracellular Ca^{2+}, in addition to PKA, but not AC, to stimulate baboon GH release [25]. The fact that combined treatment of ghrelin with an sst5 agonist increased cAMP accumulation greater than that observed with the sst5 agonist alone, also suggest these receptors interact directly or their signals converge downstream. Evidence for a direct physical interaction between sst5 and GHS-R1a has been reported in HEK293 cells transfected with both human receptors using BRET method [57]. Interestingly, this study determined in cells expressing GHS-R1a and sst5 that SST amplified ghrelin-induced increases in intracellular Ca^{2+} levels and, that GHS-R1a attenuated inhibition by SST of forskolin-induced cAMP accumulation by modifying sst5 signal transduction. However, whether heterodimers of sst5/GHS-R1a are formed in pituitary somatotropes remains to be determined.

The physiologic relevance for the biphasic effects of SST (i.e. GH suppression at moderate to high doses and GH stimulation at low doses) remains to be determined. However, based on the concentration patterns of SST released into the pituitary circulation, as determined by hypophyseal portal blood sampling in rats, pigs and sheep [76-78], it is possible that SST input to the pituitary can fall *in vivo* to levels observed to be stimulatory to GH release *in vitro* and therefore, may contribute to initiating GH pulse release. The stimulatory effect of SST may also contribute to the rise in circulating GH following discontinuation of SST infusion in humans and animal models [37, 79-85]. This "rebound" release has been attributed to degradation of circulating SST concentrations to levels sufficient to allow for enhanced GHRH neuronal activity and subsequent GH release. However, the fact that a rapid rebound in GH secretion has also been observed following SST infusion in cultured pituitary cell systems [86, 87], coupled with the clear stimulatory effect of low-dose SST observed in this and previous studies [4, 6-8, 10], suggests a component of the GH rebound effect observed *in vivo* includes degradation of circulating SST to concentrations within the stimulatory range. The stimulatory actions of SST might also be important in the fasting-induced rise in GH. It has been reported that hypothalamic SST mRNA is reduced in food-restricted sheep [88] and fasted mice [32], where in sheep this is associated with a decrease in SST levels in hypophyseal portal blood [88]. Based on our observation that low-dose SST and ghrelin evoke an additive effect on GH release *in vitro*, it is possible that the low levels of SST observed with fasting, may work in concert with the well characterized rise in circulating ghrelin levels [89] to further augment GH release. Finally, although the current study used pituitary cell cultures from normal baboons, the results may provide clues regarding the patho-physiology and treatment of GH-producing adenomas, where the current results support the hypothesis that a shift in the relative expression or activity levels of the SST receptor subtypes from inhibitory to stimulatory, may in part explain why a subset of tumors are unresponsive to conventional SST analogue therapy [90]. Clearly, much work remains to be done, in order to fully understand the importance of the dose-dependent, biphasic effects of SST on GH release. However, the current report provides solid evidence that this phenomenon is indeed real and can occur in a species relevant to humans.

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Supplemental table 1: Baboon-specific primers for amplification of transcripts SST receptors (sst1, sst2, sst3, sst4, sst5), GH, PRL and cyclophilin-A, used for qrtRT-PCR.

GENE	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
sst1	EF639291	**Sense**: AGGTAGTAAACCTGGGGGTGTG **Antisense**: AGCACGTAGCACAGGCAGATAG	**Sn** 236 **As** 446	211
sst2	EF639292	**Sense**: TGGCATCAATCAGTTCACCA **Antisense**: TACCAAGCCCCAGATTCACC	**Sn** 159 **As** 407	249
sst3	EU156181	**Sense**: CCAGCCCTTCAGTCACCA **Antisense**: CCGAAGGGCCAGTAAGACA	**Sn** 56 **As** 170	115
sst4	EU156182	**Sense**: TCTTTGTGCTCTGCTGGATG **Antisense**: AACCATAGAGTACGGGGTTGG	**Sn** 234 **As** 371	138
sst5	EF639293	**Sense**: ACTTCTTCGTGGTCATCCTCT **Antisense**: AACCTTCTGGAAGCTCTGG	**Sn** 49 **As** 146	98
GHRH-R	DQ340391	**Sense:** TCACCATCCTGGTTGCTCTC **Antisense:** GCAGCATCCTTCAGGAACAC	**Sn** 74 **As** 185	112
GHS-R	DQ340392	**Sense:** GTGTGGGTGTCCAGCATCTT **Antisense:** CACGGTTTGCTTGTGGTTCT	**Sn** 389 **As** 535	147
GH	DQ340390	**Sense**: GACCTAGAGGAAGGCATCCAAA **Antisense**: AGCAGCCCGTAGTTCTTGAGTAG	**Sn** 21 **As** 163	143
PRL	EF419886	**Sense**: CCTTCGAGACCTGTTTGACC **Antisense**: ATCTGTTGGGCTTGCTCCTT	**Sn** 14 **As** 196	183
Ciclophilin-A	DQ315473	**Sense**: CAAGACGGAGTGGTTGGATG **Antisense**: TGGTGGTCTTCTTGCTGGTC	**Sn** 351 **As** 472	122

Article V

Homologous and heterologous in vitro regulation of pituitary receptors for somatostatin, growth hormone (GH)-releasing hormone and ghrelin in a non-human primate (*Papio anubis*).

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Abstract

Secretion of GH by pituitary somatotropes is primarily stimulated by GHRH and ghrelin and inhibited by somatostatin through the activation of specific receptors (GHRH-R, GHS-R and sst1-5, respectively). In addition, we have previously shown that low doses of somatostatin also stimulate GH release in primary pituitary cultures from baboons (*Papio anubis*) and pigs via sst5. Since somatotrope responsiveness can be critically dependent on receptor regulation we sought to determine whether and how somatostatin, GHRH and ghrelin regulate the expression of their receptors in a primate model (baboon) closer to human physiology than the usual experimental rodent models. Thus, primary baboon pituitary cell cultures were treated for 4h with GHRH, ghrelin (10^{-8}M) or with high (10^{-7}M) and low (10^{-15}M) doses of somatostatin and GH release and expression levels of all receptors were measured. GHRH and ghrelin decreased expression of their respective receptors (GHRH-R, GHS-R), while both peptides increased sst1, did not affect sst2, and only GHRH decreased sst5 expression. These effects of GHRH and ghrelin were completely mimicked by forskolin (adenylate cyclase-activator) and TPA (PKC-activator), respectively, suggesting that the regulation of receptor-isoform levels by GHRH and ghrelin involved distinct signaling pathways. In contrast, high SST doses did not alter GH release and did not modify GHRH-R and GHS-R levels, but markedly increased sst1, 2, and 5 expression. Interestingly, as observed with GHRH, low doses of SST stimulated GH release and increased sst1, while decreasing sst5 and GHRH-R expression. Taken together, our data show, for the first time in a primate model, that primary regulators of somatotrope function (GHRH, ghrelin, and somatostatin) exert both homologous and heterologous regulation of the expression of their respective receptor, in a dose- and isoform-dependent manner, which likely involves distinct signaling pathways, and provide thereby an additional molecular level for finely adjusting the response of somatotropes to their main regulators.

Introduction

It is now widely accepted that regulation of growth hormone (GH) secretion is primarily exerted by an intricate interaction among three factors, GH-releasing hormone (GHRH) and ghrelin, which directly stimulate GH release through binding to their specific receptors (GHRH-R and GHS-R, respectively) located in the plasma membrane of pituitary somatotropes; and somatostatin (SST), which binds to five different receptors (sst1-5) to inhibit basal and/or GHRH/ghrelin-stimulated GH release [1-11]. In addition, studies from our group have demonstrated that SST, acting at low doses, can act as a true GH-releasing factor in primary pituitary cell cultures from baboons (a non-human primate; *Papio anubis*) and pigs [5, 12-18]. Also, it has been reported, but not emphasized in the literature, that some human GH-producing pituitary adenomas release GH in vitro in response to SST or SST receptor agonist challenge [19-23]. These divergent effects have been attributed to SST activation of distinct receptors (sst1, sst2 and sst5) which have been identified as the most highly expressed ssts within the pituitary of mammalian species (including humans, baboons and rodents [5, 14, 15, 24, 25] and are considered to be the primary mediators of the actions of SST on GH release [5, 14, 15]. Specifically, the inhibitory effect of SST is mediated primarily by activation of the sst1 and sst2 while the stimulatory effect is mediated by sst5 [5, 14, 15].

Previous studies have shown that these pituitary receptors (GHRH-R, GHS-R and sst-subtypes) differ in their ability to activate specific intracellular signaling pathways on binding of their ligand [3, 7, 26]. Thus, GHRH mainly requires activation of adenylate cyclase (AC), cAMP production and protein kinase A (PKA), whereas ghrelin predominantly leads to the activation of phospholipase C (PLC), phosphatidylinositol turnover and protein kinase C (PKC) to mediate their GH-releasing actions in primate [27] and other species [7, 28, 29], although it also requires cAMP-route activation to exert its full effect in porcine somatotropes [30]. Likewise, the GH stimulatory effect of low-dose SST and sst5-agonist is mediated through AC/cAMP/PKA and consequently, does not augment GHRH-induced GH release, but can result in additive effects when combined with ghrelin on intracellular signaling pathways [14, 15]. On the other hand, the GH inhibitory effect of high-dose SST (via sst2) seems to be

mediated through an inhibition of basal cAMP levels [14]. As recently review by Melmed´s group [3], this phenomenon, the dual dose-dependent effect of SST on cAMP, could be explained by sst-subtypes coupling to either Gαi/o or Gαs depending on the ligand binding and receptor conformation.

Also, there is emerging evidence that the relative expression level of each receptor within the target cell (i.e. pituitary) can differentially influence the sensitivity of the cells to their ligand, this being especially important in the treatment of human pituitary adenomas with SST analogs. In fact, it has been demonstrated that the therapeutic response of pituitary tumors (e.g. GH-secreting adenomas) to these analogs may be dependent on the relative expression pattern of the sst-subtypes [31-33]. However, the current knowledge on the regulation of pituitary sst-subtype expression pattern in response to its ligand is far from clear, as different species and experimental settings (mainly rodent cell lines, rat pituitary or human tumor cultures) as well as techniques (in situ hybridization, immunocytochemistry and mRNA expression) have been used, and most reports have not discriminated between different receptors subtypes [3, 5, 34-39]. Thus, a thorough study of the regulation of GH-related pituitary receptors in response to their ligands would be of high value to understand the physiological functioning of SST/ssts in normal human pituitary. Since this goal is not easily attainable in normal human pituitary samples, in the current study primary pituitary cell cultures from normal female baboons (*Papio anubis*), a primate species that closely models human physiology [27, 40-42] was used to study, for the first time, the homologous and heterologous in vitro regulation of pituitary GHRH-R, GHS-R, and sst1-5 in response to GHRH, ghelin and high and low doses of SST.

Materials and Methods

Culture reagents

Unless otherwise indicated, reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO). α-Minimum essential media (α-MEM), HEPES, horse serum and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY). Ghrelin was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Somatostatin-14 (SST) was purchased from Sigma-Aldrich and Phoenix Pharmaceuticals. Subtype selective agonists for sst1 (L-797,591), sst2 (L-779,976), sst3 (L-796,778), sst4 (L-803,087) and sst5 (L-817,818) were generously provided by Merck & CO., INC. (Whitehouse Station, NJ) [43].

Animals and pituitary collection

Pituitaries were obtained and dispersed into single cells for culture (see below) from random cycling female baboons (*Papio anubis*, 7-14 years of age) as previously described [14, 27, 41, 44, 45]. These animals represent control animals from studies conducted by other University of Illinois at Chicago investigators, where all studies were approved by the Institutional Animal Care and Use Committee.

Primary pituitary cell culture

Anterior pituitaries were dispersed into single cells by enzymatic and mechanical disruption, as previously described [14, 27, 41, 44, 45]. Dispersed cells were plated onto 24-well tissue culture plates at a density of 200,000 cells/well in 0.5 ml of basic medium containing 10% horse serum. After a 36-48h of incubation (37C; 5%CO2), medium was removed and cells were pre-incubated for 1h in fresh, warm (37C) serum-free medium to stabilize basal hormone secretion. Then, medium were replaced for an additional 4h (3-4 wells/treatment/experiment) with serum-free medium alone (controls) or containing human SST (at a 100nM or 0.001pM-doses which have been previously identified to exert a maximal inhibitory or stimulatory effect on basal and/or stimulated-GH release, respectively [14]), GHRH (10-8M), ghrelin (10-8M), selective agonists for sst1-4 (10-7M) and sst5 (10-7 and 10-11 M), forskolin (a direct activator of AC; 1μM) or phorbol 12-myristate 13-acetate (TPA; a direct activator for PKC; 0.1μM). Then, media was removed and frozen for subsequent analysis of hGH levels and total cellular recovered for determination of mRNA levels, as described below. The doses of all peptides/compounds used in this study were selected according to previous studies [14, 27].

Hormone analysis

Culture media was recovered, centrifuged (2000g/5 min) and stored at –80C for subsequent analysis of GH concentrations using a commercial hGH ELISA kit (DSL, Webster, Texas or DRG, Mountainside, NJ].

RNA isolation, reverse transcription (RT) and quantitative real-time PCR (qrtRT-PCR)

Total RNA from primary pituitary cell cultures was extracted, quantified and reverse-transcribed, as previously described [14, 27, 44, 45]. cDNA obtained was treated with

Table 1. Baboon-specific primers for somatostatin receptor subtypes (sst1-5), growth hormone-releasing hormone receptor (GHRH-R), ghrelin receptor (GHS-R) and cyclophilin-A, used for quantitative real-time, RT-PCR.

GENE	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
sst1	EF639291	**Sense:** AGGTAGTAAACCTGGGGGTGTG **Antisense:** AGCACGTAGCACAGGCAGATAG	**Sn** 236 **As** 446	211
sst2	EF639292	**Sense:** TGGCATCAATCAGTTCACCA **Antisense:** TACCAAGCCCCAGATTCACC	**Sn** 159 **As** 407	249
sst3	EU156181	**Sense:** CCAGCCCTTCAGTCACCA **Antisense:** CCGAAGGGCCAGTAAGACA	**Sn** 56 **As** 170	115
sst4	EU156182	**Sense:** TCTTTGTGCTCTGCTGGATG **Antisense:** AACCATAGAGTACGGGGTTGG	**Sn** 234 **As** 371	138
sst5	EF639293	**Sense:** ACTTCTTCGTGGTCATCCTCT **Antisense:** AACCTTCTGGAAGCTCTGG	**Sn** 49 **As** 146	98
GHRH-R	DQ340391	**Sense:** TCACCATCCTGGTTGCTCTC **Antisense:** GCAGCATCCTTCAGGAACAC	**Sn** 74 **As** 185	112
GHS-R	DQ340392	**Sense:** GTGTGGGTGTCCAGCATCTT **Antisense:** CACGGTTTGCTTGTGGTTCT	**Sn** 389 **As** 535	147
Ciclophilin-A	DQ315473	**Sense:** CAAGACGGAGTGGTTGGATG **Antisense:** TGGTGGTCTTCTTGCTGGTC	**Sn** 351 **As** 472	122

Ribonuclease H (1U; Fermentas) and duplicate aliquots (1µl) were amplified by quantitative real-time RT-PCR (qrtRT-PCR) using the Stratagene Brilliant SYBR green QPCR Master Mix. Details regarding the development, validation and application of qrtRT-PCR to measure expression levels of different baboon transcripts have been recently reported by our laboratory [14, 27]. Briefly, to determine the starting copy number of cDNA, RT samples were PCR amplified, and the signal (Ct) of each sample was compared with that of a standard curve run in the same PCR plate. Standard curves consisted of 1, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 copies of synthetic cDNA template for each of the transcripts of interest. In addition, total RNA samples that were not reversed transcribed and a no DNA control were run on each plate to control for genomic DNA contamination and to monitor potential exogenous contamination, respectively. Specific sets of primers used in this study to measure expression levels of baboon sst1-sst5, GHRH-R and GHS-R are shown in supplemental table-1. To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (used as housekeeping gene), where cyclophilin A mRNA levels did not significantly vary between experimental groups (data not shown).

Statistical analysis

In order to normalize mRNA values within each treatment and minimize intra-group variations (likely due to variations in age, body conditions, and/or reproductive status), the values obtained were compared to vehicle-treated controls (set at 100%) and the results are reported as the mean ± SEM in all experiments. Each treatment group was tested in a minimum of 3 separate pituitary cultures each prepared from a different animal, and within each pituitary cell preparation (experiment) treatments were replicated in at least 3-4 wells. Differences between treatment groups were assessed by t-student`s test and/or by analysis of variance (on-way ANOVA), followed by Newman-Keuls post-test for multiple comparisons. $p<0.05$ was considered significant. All statistical analyses were performed using GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD).

Results

Effects of SST on sst1-5, GHRH-R and GHS-R mRNA levels

We treated primary pituitary cell cultures with two doses of SST (high, 10^{-7}M; and low, 10^{-15}M), which have been previously shown to exert different GH secretory responses in baboon and porcine somatotropes [12, 13, 15, 16, 18]. Specifically, these were a high SST dose which has been demonstrated that does not alter basal GH release but inhibits GHRH and ghrelin-stimulated GH release and a low SST dose that increases basal GH release, as was confirmed in the present study (Fig.-1A). As shown in Fig.-1B, treatment of baboon pituitary cell cultures for a 4-h period with 10^{-7}M SST increased sst1, sst2, and sst5 expression but did not alter sst3, sst4 (data not shown), GHRH-R or GHS-R. Interestingly, the low dose of SST also increased sst1 expression,

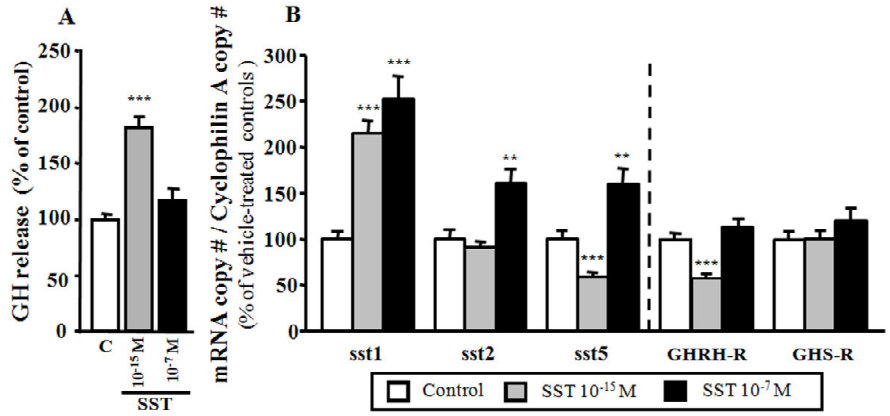

Figure 1. Effects of SST 10-15M and SST 10-7M (4h) on GH release **(A)** and, on the expression of sst1, sst2, sst5, GHRH-R and GHS-R as assessed by qrtRT-PCR **(B)** in primary pituitary cell cultures from baboon (Papio anubis). Values are expressed as percentage of vehicle-treated controls (set at 100% within experiment), and represent the mean ± SEM of four independent experiment (3-4 well/treatment/experiment). Asterisks indicate values tha significantly differ from their respective controls assessed by Student`s t test (*, p<0,05; **, p<0,01; ***, p<0,001).

had no effect on sst2 or GHS-R but, significantly inhibited sst5 and GHRH-R mRNA levels. These results suggest that SST, depending of the dose applied, exert both homologous and heterologous regulation on the expression of its own receptors or GHRH-R.

Effects of GHRH, ghrelin, forskolin and TPA on sst1-5, GHRH-R and GHS-R mRNA levels

As previously reported [27, 41], we observed that GHRH and ghrelin (10^{-8}M) markedly increased GH release in baboon pituitary cultures (Fig.-2A). As shown in Fig.-2B, GHRH and ghrelin decreased the expression of their own receptors, GHRH-R and GHS-R, whereas they did not alter the expression of the receptors for each other. Interestingly, both GHRH and Ghrelin up-regulated sst1 but did not alter sst2 expression. However, GHRH, but not ghrelin, decreased sst5 mRNA levels (Fig.-2B).

Interestingly, it should be noted that these effects of the acute GHRH treatment on the expression of all these receptors were fully mimicked by an activator of adenylate cyclase (forskolin), while the effects of ghrelin treatment on the same receptors were mimicked by a protein kinase C activator (TPA) (Fig.-2C), thereby suggesting that the homologous and heterologous regulation of receptor-isoform levels by GHRH and ghrelin involves distinct signaling pathways. Finally, expression of sst3 and sst4 was not altered by GHRH, ghrelin, forskolin or TPA (data not shown).

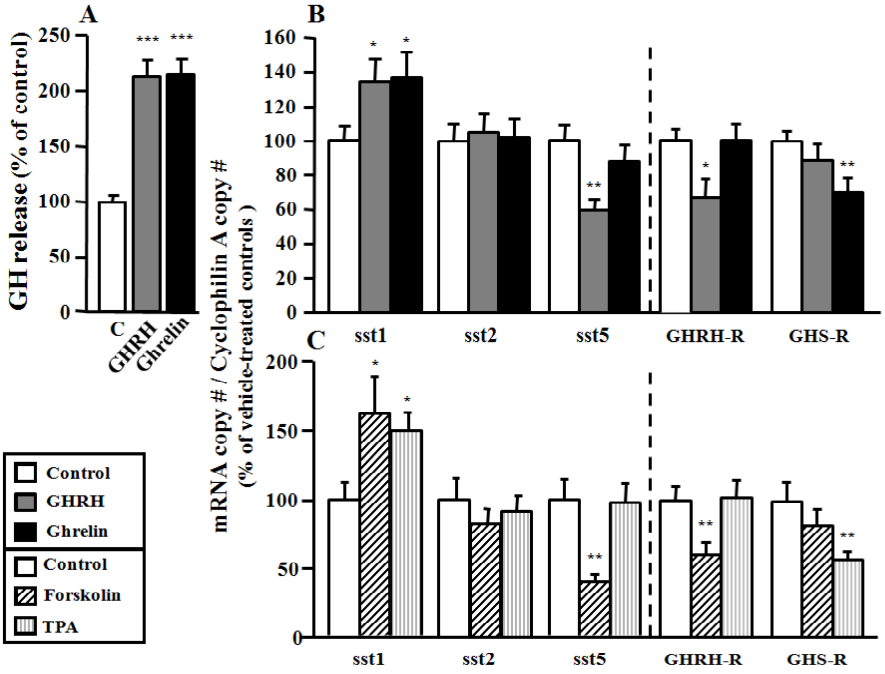

Figure 2. Effects of GHRH and Ghrelin (4h) on GH release **(A)** and, on the expression of sst1, sst2, sst5, GHRH-R and GHS-R as assessed by qrtRT-PCR **(B)** in primary pituitary cell cultures from baboon (Papio anubis). Effect of forskolin (a direct activator of AC) and TPA (a direct activator of PKC) (4h) on the expression of sst1, sst2, sst5, GHRH-R and GHS-R as assessed by qrtRT-PCR **(C)**. Values are expressed as percentage of vehicle-treated controls (set at 100% within experiment), and represent the mean ± SEM of four independent experiment (3-4 well/treatment/experiment). Asterisks indicate values tha significantly differ from their respective controls assessed by Student`s t test (*, p<0,05; **, p<0,01; ***, p<0,001).

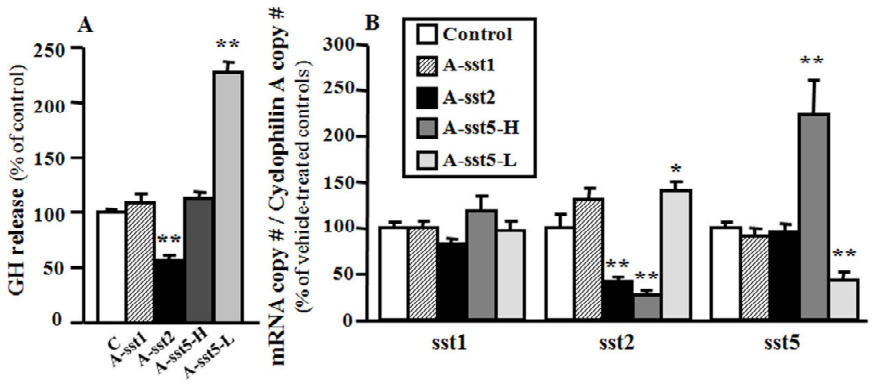

Figure 3. Effects of subtype selective agonists for sst1, sst2, and sst5 at 10-7M (A-sst1, A-sst2 and A-sst5 H) and at 10-11M (A-sst5 L) (4h) on GH release **(A)** and, on the expression of sst1, sst2 and sst5 **(B)**, in primary pituitary cell culture from baboon (Papio anubis). Values are expressed as percentage of vehicle-treated controls (set at 100% within experiment), and represent the mean ± SEM of four independent experiment (3-4 well/treatment/experiment). Asterisks indicate values tha significantly differ from their respective controls assessed by Student`s t test (*, p<0,05; **, p<0,01)

Effect of selective sst1, sst2 and sst5 agonists on sst1-5 mRNA level

As we have previously demonstrated that the inhibitory effect of SST at high dose is primarily mediated by activation of the sst1 and sst2 while the stimulatory effect of SST at low doses is mediated by sst5 [14, 15] and based on the above results indicating that sst3 and sst4 expression was not altered by any treatment employed, we used selective agonist for sst1 and sst2 at high (10^{-7}M) dose and sst5 at high or low (10^{-7} or 10^{-11} M) doses to determine whether activation of specific sst-receptors subtypes may influence the expression of other sst-subtypes. As previously reported [14], we found that high-dose of sst1 or sst5 agonists did not alter basal GH release (Fig.-3A). However, high-dose of sst2-agonist inhibited basal GH secretion while, low-dose of sst5 agonist markedly increased GH output (Fig.-3A). In terms of regulation of transcript levels, we found that sst1 expression was not altered by any sst-agonist (Fig.-3B) however, mRNA levels for sst2 were down-regulated by high-dose of sst2 and sst5 agonist while, a modest albeit significant increase was observed when low-dose of sst5-agonist were applied (Fig.-3B). On the other hand, sst5 expression was not altered by high-dose of sst1 and sst2 agonists but surprisingly, sst5 mRNA levels were oppositely regulated by high and low dose of sst5-agonist (up- and down-regulation, respectively) (Fig.-3B).

Discussion

Most of our current understanding on the direct effects of SST, GHRH and ghrelin on the expression of their pituitary receptors has been derived from studies conducted in non-human primate species (mainly rodent cell lines and rat pituitary) or human tumor preparations, and has not led to generally accepted model [1, 3, 5, 7-9, 31]. Availability of a specific set of sst-subtypes, GHRH-R and GHS-R at the cell surface, which critically define the ability of somatotropes to respond to their main regulators, can be influenced by their ligands through different mechanisms, including receptor internalization and desensitization, as well as regulation of gene expression, which, in the case of SST, is subtype-specific and dose-dependent [1, 3, 5, 6]. However, the direct pituitary effects of SST, GHRH and ghrelin on all sst-subtypes, GHRH-R and GHS-R in normal adult humans remain largely unknown. Due to their close homology with humans at the physiologic and genomic levels, the baboon (*Papio anubis*) has emerged in recent years as a valuable non-human primate model to study different levels of human physiology (including pituitary function) [14, 27, 41, 45, 46]. Accordingly, the present study was conducted using primary pituitary cell cultures from normal adult female baboons as a model to study the impact of these endogenous ligands on the expression of their receptors and each other receptor (i.e. homologous and heterologous regulation).

Our results demonstrate that high dose of SST can up-regulate sst1, sst2, and sst5 expression in baboon primary pituitary cell cultures. These results are similar to those found in other in vitro models of non-primate species (i.e. pigs and rats) and pituitary cell lines (GH3) [34, 35, 37, 39, 47], and also, with an in vivo mouse model of enhanced hypothalamic SST expression (the MT-hGHRH), wherein sst2 and 5 mRNA levels were found to be upregulated [38], thus suggesting that the regulation of sst2 and sst5 (which are the main receptors involved in GH release) [5] by high dose of SST is conserved across species. Interestingly, treatment of baboon pituitary cell cultures with a low dose of SST (which increases GH release) partially evoked the effect observed in a previous report on porcine primary pituitary cell cultures [47], in that it similarly increased sst1 and did not alter sst2 expression. However, low dose of SST oppositely regulated sst5 expression as compared with high dose of SST, since it markedly decreased sst5 expression in baboon, but not pig [47], pituitary cultures. We

next examined whether SST could also regulate expression of GHRH-R and GHS-R in the baboon pituitary. This showed that SST did not alter the expression of GHS-R at any of the doses tested, which is clear contrast to the up-regulation in GHS-R mRNA levels previously observed in pig pituitary cultures [36]. Interestingly, our results indicate that low, but not high, doses of SST potently decreased GHRH-R mRNA levels, which closely resemble the results found in pig [36], and thereby suggest that heterologous regulation of GHRH-R by low dose of SST is conserved across species. Taken together, these results indicate that SST not only regulates its own receptors synthesis but also GHRH-R expression in the pituitary and, this regulation seems to be dose- and species-dependent as well as specific for the sst-subtypes.

Studies using sst-subtype specific agonists [43, 48] revealed that a high dose of sst2-agonist (which inhibited basal GH release) causes a down-regulation of sst2 expression that is in clear contrast to that exerted by high dose of SST. These opposite events may be explained by a differential ligand (SST vs. sst2-agonist)-induced activation of this receptor, since it has been previously observed that different somatostatin analogs may induce distinct subcellular expression pattern of sst-subtypes and/or different conformations of the receptor/ligand complex, preferentially coupled to either receptor signaling or receptor endocytosis [49, 50]. In fact, SST and sst2-agonist may activate distinct signalling mechanisms or molecular events (i.e. homo- or heterodimerization between sst-subtypes) [51], where the differential activation of these intracellular signalling pathways and/or association between sst-subtypes would have variable effects on sst2 expression. In support of this notion, we have recently shown that an intact MAPK activity is required for sst2-agonist but not SST-mediated regulation of GH release in baboon primary pituitary cell cultures [14]. Moreover, it has been shown that treatment of SST and sst2-agonist promote the formation of sst2-sst5 heterodimer complexes in an heterologous cellular system (HEK293 and CHO-K1 cells), however the subsequent MAPK signalling and AC inhibition was significantly higher with sst2-agonist than with SST treatment [52, 53]. Furthermore, it has been demonstrated that sst2 is retained (not internalized) at the plasma membrane in response to sst2-agonist challenge in cells that co-express sst2 and sst5 (as occurs in somatotropes [54, 55]) whereas endogenous SST potently internalize and desensitize sst2 [52, 53]. Although caution should always be taken in the interpretation of data generated by the use of SST agonists, these results shed new light on the complex relationships among sst subtypes and their ligands, which may result useful to study the properties of therapeutically relevant SST analogs, and pave the way for future investigations.

Interestingly, we observed that treatment with high or low dose of sst5-agonist (which do not alter or increase basal GH release, respectively) ([14] and present study) induced opposite effects on sst2 and sst5 expression. Thus, high doses of sst5-agonist reduced sst2 and increased sst5 expression, while low doses of sst5 agonist increased sst2 and decreased sst5 mRNA levels. These observations may not be atypical and could be of physiological relevance for the somatotropes, in that the activation of sst5 by low doses of SST or sst5-agonists markedly increase GH release in baboons and pigs ([14, 15] and present study]) and therefore, somatotropes may respond to this challenge by increasing the amount of the main receptor involved in the inhibition of somatotrope-function, sst2, and/or by decreasing that of the stimulatory receptor (sst5), in order to inhibit stimulated-GH release [5, 14, 15]. On the other hand, activation of sst5 by high doses of SST may activate distinct signalling mechanisms or molecular events (i.e. heterodimerization of sst5 and sst2) [52-55] that may be interpreted as an overall inhibitory input by somatotropes, which may thus respond by increasing the expression of the sst5 and decreasing sst2 mRNA levels. In support to this idea, our laboratory has reported that low dose of the sst5-agonist used in the present study (L-817,818) as well as low dose of SST signal through AC/cAMP/PKA to increase basal GH release [14]. Altogether, our results suggest that this opposite regulation of sst2 and sst5 in response to different doses of sst5-agonist might mediate an inner counterbalance of somatotrope cells to facilitate or avoid excessive GH release. Moreover, these results may be (patho)-physiologically relevant in humans, in that we have shown that the amount and ratio between pituitary sst2 and sst5 expression in human with GH-secreting adenomas might be a key factor for the hormonal control of these patients in response to octreotide (a preferential agonist for sst2 and sst5) treatment [32, 33].

We also examined the impact of acute (4h) treatment of GHRH and ghrelin on regulating receptor synthesis in adult baboon pituitary cell cultures. Our results revealed that, GHRH-R and GHS-R were down-regulated by its endogenous ligand which is consistent with previous reports in other species (including the baboons, pigs and rodents) [27, 36, 56-59], showing that the synthesis of these receptors can be rapidly

down-regulated after binding of it ligands. However, it should be noted that the homologous regulation of GHRH-R and GHS-R by its own ligands may be dependent of the time of incubation, culture conditions, species, and age studies [36, 56, 59-63].

In addition of the inhibitory effect induced by both GHRH and ghrelin on the expression of their respective receptors in baboon pituitary cell cultures, we observed that these peptides exerted a heterologous regulation in sst-subtypes. Specifically, both GHRH and ghrelin increased sst1 and did not alter sst2 expression while, only GHRH was able down-regulated sst5 expression. Interestingly, we noticed that the regulation of all sst-subtypes, GHRH-R and GHS-R was virtually identical in response to GHRH and low dose of SST, suggesting that these events may be mediated through a common signalling pathway. In fact, we have recently reported that the effects of GHRH and low dose of SST on stimulating baboon GH release are mainly mediated through an increase in intracellular cAMP levels [14]. Therefore, in an attempt to better understand the relative contribution of elevated intracellular cAMP levels in the synthesis of pituitary receptors, we sought to determine whether forskolin could exert the same effect than GHRH or low dose SST on sst1, sst2, sst5, GHRH-R and GHS-R. Our results clearly indicate that an increase in intracellular cAMP levels seems to be the responsible of the changes observed in the expression of all these receptors after GHRH and low dose SST challenge. It is also interesting to note that the effects of ghrelin on the synthesis of all the pituitary receptors analyzed in this study were mimicked by TPA (phorbol esters; an activator of PKC), suggesting that this signalling pathways is required not only for the GH releasing effect of ghrelin [36] but also for the regulation of the synthesis of these pituitary receptors in response to ghrelin. Interestingly, our study uncovered unique species-specific differences in the regulation of pituitary receptor synthesis in response to SST, GHRH, ghrelin, which may probably be related to the differences in the intracellular signalling pathways activated by these endogenous ligands in pigs, where cAMP activation is also required by ghrelin to exert its GH-releasing action [17, 29, 30, 36, 47, 64-66] and baboons, in which cAMP does not play such a role [14, 27].

In summary, our data show for the first time in a primate model, that, in addition to the well-defined role of SST, GHRH and ghrelin in modulating GH release, these primary regulators of somatotrope function act through distinct signaling pathways to exert both homologous and heterologous regulation of receptor expression, a mechanism that may contribute significantly to modulate the somatotrope response to acute ligand stimulation and thus, would provide an additional layer of complexity to the regulatory mechanisms required to maintain circulating GH levels in a normal range.

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Article VI

Kisspeptin regulates gonadotroph and somatotroph function in non-human primate pituitary via common and distinct signaling mechanisms

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Kisspeptins (Kps) have emerged as key players in the control of reproductive-axis function, where they operate as primary regulators of hypothalamic GnRH release. In addition, recent data indicates that Kps can also directly act on the pituitary to stimulate luteinizing-hormone (LH) and growth-hormone (GH) release in primary pituitary cell culture prepared from rats, cows and sheep. We present herein evidence that Kps (specifically Kp-10) can also stimulate LH and GH release in primary pituitary cell cultures prepared from female baboons (Papio anubis), a species that more closely models human physiology. The stimulatory effect of Kp-10 on LH and GH release was dose- and time-dependent and enhanced the hormonal responses to their major regulators (GnRH for LH; GHRH/ghrelin for GH) without affecting the release of other pituitary hormones (TSH, FSH, ACTH, PRL). Use of pharmacologic intracellular signaling blockers indicated Kp-10 signals through PLC, PKC, MAPK and intracellular Ca2+ mobilization, but not AC, PKA, extracellular Ca2+ influx (through L-type channels) or NOS, to stimulate both LH and GH release. Interestingly, blockade of mTOR or PI3K activity fully abolished the stimulatory effect of Kp-10 on LH, but not GH release. Of note, estradiol enhanced the relative LH-response to Kp-10, alone or in combination with GnRH. In sum, our data are the first to provide evidence that, in a primate model there is a functional Kp-signaling system within the pituitary, which is dynamically regulated and may contribute to the direct control of gonadotropic- and somatotropic-axes. (***Endocrinology In press***)

Kisspeptins (Kps) are the peptide products of the *Kiss1* gene, which signal through the G protein-coupled receptor *Kiss1r* (also known as GPR54) to regulate reproductive function. The importance of Kps/GPR54 is emphasized by the observation that some forms of hypogonadotropic hypogonadism are caused by inactivating mutations of *GPR54* in humans and rodents (1, 2). Subsequent studies, conducted in diverse mammalian and non-mammalian species, have confirmed that Kps are essential gatekeepers of proper reproductive maturation and function, including brain sexual differentiation, puberty onset and neuroendocrine control of gonadotropin secretion and its gating by metabolic and seasonal cues (3).

It is clear that Kps act at the level of the hypothalamus to stimulate gonadotropin-releasing hormone (GnRH) release. Detailed neuroanatomical studies in rodents, sheep, primates and fish led to the identification of discrete populations of hypothalamic neurons that express *Kiss1*/kisspeptin; and presence of functional *Kiss1r* have been documented in GnRH neurons. Specifically, in mammals, a prominent population of *Kiss1* neurons has been demonstrated in the arcuate nucleus in both sexes (4, 5). In addition, in rodents another group of kisspeptin neurons are located in the anteroventral periventricular nucleus and adjacent areas, where this neuronal population seems to be more abundant in females (4, 5). In fact, direct appositions between *Kiss1* terminals in the AVPV and GnRH neurons have been described in rodents (6). Similarly, kisspeptin- and GnRH-axons have been found in close association within the median eminence of monkeys (7). These findings, coupled with an abundance of convincing functional data, have led to the notion that *Kiss1* neurons within the hypothalamus are

pivotal afferents in the circuitry governing GnRH secretion, where they operate as major nodal points for the integration and transmission of key regulatory signals, from sex steroids to metabolic hormones (3). Evidence is also accumulating showing *Kiss1* and *Kiss1r* are expressed in other brain (extra-hypothalamic) and peripheral tissues, thus suggesting additional sites of action, if not different biological roles, for Kps.

In this regard, the possibility that Kps could exert direct modulatory effects on gonadotropin secretion at the pituitary level was put forward by initial studies of characterization of the pharmacological effects of Kps on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release in the rat (8). Since then, several groups have documented the ability of Kps to elicit LH secretion by pituitary tissue *in vitro*, in rodent, bovine and ovine species (9-13). In addition, Kps have been detected in the portal blood of the pituitary in sheep (12), and recent immunohistochemical studies in the monkey suggest a potential direct effect on the pituitary (7). Furthermore, expression of *Kiss1* and *Kiss1r* mRNAs has been documented in the pituitary of rats, sheep and humans, under the control of GnRH and/or sex steroids (14-17). Collectively, these data suggest a plausible functional role of Kp signaling at the pituitary level. Admittedly, however, some other studies have been unable to detect any direct action of Kps in the pituitary of rats (18, 19). Moreover, the physiological relevance of such direct effects, at least regarding their involvement in the induction of the pre-ovulatory LH surge, has also been questioned in sheep (12). Evidence also suggests that the pituitary actions of Kps might involve the modulation of other neuroendocrine axes, such as the somatotroph system (9, 10, 20, 21), but this action also remains controversial.

In the present work we aimed to verify the direct pituitary actions of Kps on hormone release using the female baboon as a model. We hypothesized that kisspeptin signaling in pituitary cells may play a role in the fine tuning of the gonadotropic axis, complementary to its dominant, pivotal function at the hypothalamus. In addition, based on our previous data in the rat (9, 10, 20, 21), we considered the possibility that kisspeptin may directly regulate pituitary somatotroph function. To test these hypotheses, both LH and GH secretory responses were monitored in primary cell cultures of female baboon pituitaries after kisspeptin-10 (Kp-10) treatment, alone or in the presence of well-known modulators of gonadotroph (GnRH) or somatotroph [growth hormone-releasing hormone -GHRH-, somatostatin -SST- or ghrelin) function. Furthermore, since there is solid evidence indicating that Kps can activate a wide variety of intracellular signals via GPR54 in primary cultures as well as heterologous cell models transfected with GPR54 (3, 22), the relative contribution of major intracellular signaling pathways to Kp-10 mediated LH and GH release were studied using pharmacological blockers.

Material and Methods

Culture reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Kp-10, GnRH, GHRH, ghrelin and SST were purchased from Sigma and Phoenix Pharmaceuticals (Burlingame, CA). Minimum essential media (α-MEM), HEPES, horse serum and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY). Inhibitors of intracellular signaling pathways were purchased from Cayman chemical (U73122 and L-name; Ann Arbor, MI) or Tocris bioscience (Rapamycin; Ellisville, MI).

Animals and tissue collection

Pituitaries were obtained from randomly cyclic female baboons (*Papio anubis*, 7-12 years of age) within 15 minutes after sodium pentobarbital overdose. The selected baboons represent control animals from a breeding colony. All procedures were conducted under the Institutional Animal Care and Use Committee at the University of Illinois, Chicago. After the animals were killed, pituitaries were immediately excised and placed in sterile cold (4C) basic media consisting of α-MEM, 0.15% bovine serum albumin, 6 mM HEPES, and 10 IU/ml penicillin and 10 μg/ml streptomycin. Pituitaries were then washed twice in fresh media and divided into smaller fragments with surgical blades. Some fragments were rapidly frozen in liquid nitrogen and stored at -80C until RNA isolation (see below for details), while the remaining fragments were dispersed into single cells for culture as described below. Dissection of the hypothalamic area was done consistently in every animal by excising a central region of the hypothalamus with a cube form (parallel lateral and coronal cuts separated 5 mm from each other,

centered around midline), which provided a tissue fragment of approximately 0,15 cm3, likely containing several distinct neuronal nuclei.

Primary pituitary cell culture

Anterior pituitaries were dispersed into single cells by enzymatic and mechanical disruption, as previously described (23-25). Cells were plated onto 24-well tissue culture plates at 200,000 cells/well density in 0.5 ml of basic medium containing 10% horse serum. After a 48h-incubation (37C), medium was removed and cells were pre-incubated for 1h in fresh, warm (37C) serum-free medium to stabilize basal hormone secretion. Following the pre-incubation period, medium was replaced with serum-free medium containing treatments. Experiments specifically tested the effects of: **A**) Kp-10 alone (from 10^{-14} to 10^{-6} M) for 4h (dose-response experiment); **B**) Kp-10 alone (10^{-8} M) for 30 min or 4-, 12-, 24-, 48-h (time-course experiment); or **C**) Kp-10 alone (10^{-8} M) or in combination with GnRH, GHRH, ghrelin (10^{-8} M) or SST (10^{-7} M) for 4h. These doses were selected according to previous studies (23). In addition, in experiment **D**, in order to study the intracellular signaling pathways involved in the actions of Kp on pituitary function, medium containing the inhibitors of key intracellular signaling pathways were added following the 1h pre-incubation period (medium alone was used in the vehicle-treated controls). Ninety minutes later, the medium was replaced with medium alone (vehicle) or containing the selected inhibitor combined with Kp-10 (10^{-8}M), and incubated for an additional 4h period. Finally, in experiment **E**, in order to study the effect of sex steroids on Kp-10 mediated hormone release, cells were preincubated with medium containing estradiol (E2; 10nM) for 36h, before the day of the experiment in the presence of serum (medium without E2 was used in the vehicle-treated controls). After pre-incubation, medium was removed and cells were pre-incubated for 1h in fresh warm (37C) serum-free medium (with or without E2) to stabilize basal hormone secretion. Then, the medium was replaced with medium containing Kp-10 (10^{-8}M, with or without E2) and incubated for four additional hours. In all experiments, after the corresponding incubation period medium was collected for hormone analysis (see below). Total RNA was extracted from selected cultures treated with Kp (10^{-8} M) for expression analysis of pituitary hormone transcripts. Controls consisted of cells cultured in serum-free basic medium. Each treatment was repeated at least 3 times on different pituitary cell preparations (3-4 wells/treatment/experiment).

Hormone analysis

Culture medium was recovered, centrifuged (2000g/5 min) and stored at –80C for subsequent analysis of LH, FSH, GH, aderocorticotropic hormone (ACTH), prolactin (PRL) and thyroid-stimulating hormone (TSH) concentrations using human commercial ELISAS [DSL, Webster, Texas (for GH, reference: DSL-10-19100) or DRG, Mountainside, NJ (reference numbers: EIA-1289, EIA-1787, EIA-1288, EIA-1291, EIA-3647 and EIA-1790 for LH, GH, FSH, PRL, ACTH and TSH, respectively)] following the manufacturer's instruction. All information regarding specificity, detectability and reproducibility for each of the assays can be accessed at the websites of the indicated companies.

RNA isolation, reverse transcription (RT) and real-time PCR (rtRT-PCR)

Total RNA from primary pituitary cell cultures and whole tissue was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) with deoxyribonuclease treatment, as previously described (23-25). The amount of RNA recovered was determined by the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA was reverse-transcribed in a 20µl volume using random hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). The cDNA obtained was treated with Ribonuclease H (1U, MRI Fermentas) and duplicate aliquots (1µl) were amplified by real-time RT-PCR (rtRT-PCR) using the Stratagene Brilliant SYBR green QPCR Master Mix. Details regarding the development, validation and application of rtRT-PCR to measure expression levels of different baboon transcripts, including *cyclophilin A* (used as a housekeeping gene), have been recently reported by our laboratory (23-25). New baboon sequences obtained in the present study (LH, FSH, TSH and *Kiss1r*) were submitted to GenBank. Primer sets for baboon *LH*, *FSH*, *GH*, *ACTH*, Proopiomelanocortin *(POMC)*, *PRL*, *TSH* and *cyclophilin A* used in this study, as well as the GenBank accession numbers, are provided in Supplemental Table 1.

Statistical analysis

In order to normalize mRNA values within each treatment and minimize intra-group variations, the values obtained were compared to vehicle-treated controls (set at 100%) and the results are reported as the mean ± SEM in all experiments. Each treatment group was tested in a minimum of 3 separate pituitary cultures each prepared from a different animal, and within each pituitary cell preparation (experiment) treatments were replicated in at least 3-4 wells. The entire study was conducted on pituitaries from 11 baboons collected over a 3 year period. Data were

assessed for heterogeneity of variance, and if found, values were log-transformed. Differences between treatment groups were assessed by analysis of variance (1-way or 2-way ANOVA) with repeated measures, followed by Fisher's test for multiple comparisons. $p<0.05$ was considered significant. All statistical analyses were performed using GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD).

Results

Baboon primary pituitary cell cultures as putative model for Kp actions on human pituitary

Pituitaries from female baboons expressed *Kiss1r* at high levels compared to those found in the central region of the hypothalamus [the putative site of action of kps; (984±197 vs. 530±248 copies/0.05µg total RNA, respectively)] (8). Comparison of partial baboon mRNA sequences of all pituitary hormone genes [generated in this and previous studies (23-25)] and of *Kiss1r* with the corresponding human sequences revealed a close homology [*LH* 98%, *GH* 98%, *FSH* 99%, *PRL* 97%, *POMC* 97%, *TSH* 100% and *Kiss1r* 98%), with a higher degree of divergence vs. *Kiss1r* sequences from non-primate species (88-90% homology when compared with transcripts of pig, rat, mouse, and sheep).

To confirm that primary pituitary cells of baboons maintain a differentiated phenotype after dispersion and culture, absolute mRNA levels (copy numbers/0.05 µg total RNA) of *LH*, *GH*, *FSH*, *PRL*, *POMC*, *TSH*, *Kiss1r* and *cyclophilin A* (used as a housekeeping gene) were compared between whole tissue extracts and extracts prepared from pituitary cultures 4 h after incubation in serum-free medium and results are shown in Table-1. Transcript levels did not vary significantly between *in vivo* and *in vitro* samples, indicating that the cell preparation and culture conditions did not impact the expression of pituitary hormone genes or *kiss1r*. Together, these results support that the culture system used allows for the maintenance of correct functions of the different pituitary cell types (gonadotrophs, somatotrophs, lactotrophs, corticotrophs and thyrotrophs), and serves as a positive control that accurate quantitative real-time RT-PCR measures reflect physiologically relevant differences in gene expression.

TABLE 1. Absolute cDNA copy number/0.05µg total RNA of gene transcripts in the whole pituitary versus primary pituitary cell cultures (control groups) of female baboons, as determined by quantitative real-time RT-PCR.

Gene	Whole pituitary (Copy # ± SEM)	Pituitary cell cultures (Copy # ± SEM)
LH	18.683 ± 3.429	20.345 ± 4.122
GH	339.800 ± 88.456	298.362 ± 42.639
FSH	58.770 ± 33.607	42.792 ± 12.381
PRL	2.183.973 ± 699.518	1.893.283 ± 159.750
POMC	32.168 ± 11.223	35.271 ± 8.391
TSH	54.985 ± 32.007	41.958 ± 7.830
Kiss1r	984 ± 197	1.163 ± 241
CycloA	146.731 ± 20.648	166.320 ± 12.381

Values represent means ± SEM [n=12 separate whole pituitary extracts and n=5 separate primary pituitary cell cultures (3-5 wells/experiment)]

Direct effect of Kps on pituitary hormone release

Incubation of cultured baboon pituitary cells with increasing doses of Kp-10 (Kp) for 4h revealed clear stimulatory effects on LH and GH release in a concentration-dependent manner (at doses equal to or above 10^{-12} M; Fig-1A1 and 1B2, respectively). The lowest dose of Kp that caused a maximal increase of both LH and GH secretion was 10^{-8} M (247 ± 21 and 170 ± 4 % as compared to controls set at 100%, respectively). Accordingly, a stimulatory concentration of 10^{-8} M Kp was chosen to further analyze the action of the peptide on baboon LH and GH release.

Treatment with Kp for different incubation times (from 30min to 48h) revealed a stimulatory effect on LH release between 30min and 24h (Fig-1B1). Whereas, the stimulatory effect of Kp on GH release was observed between 4 and 24h of incubation (Fig-1B2). In both cases, hormone release was no longer significantly increased after 48h of treatment. Interestingly, Kp was able to increase *LH* mRNA levels between 12h and 48h after the exposure, while it was able to significantly stimulate *GH* expression only after 24h of incubation (Fig-1C1 and 1C2).

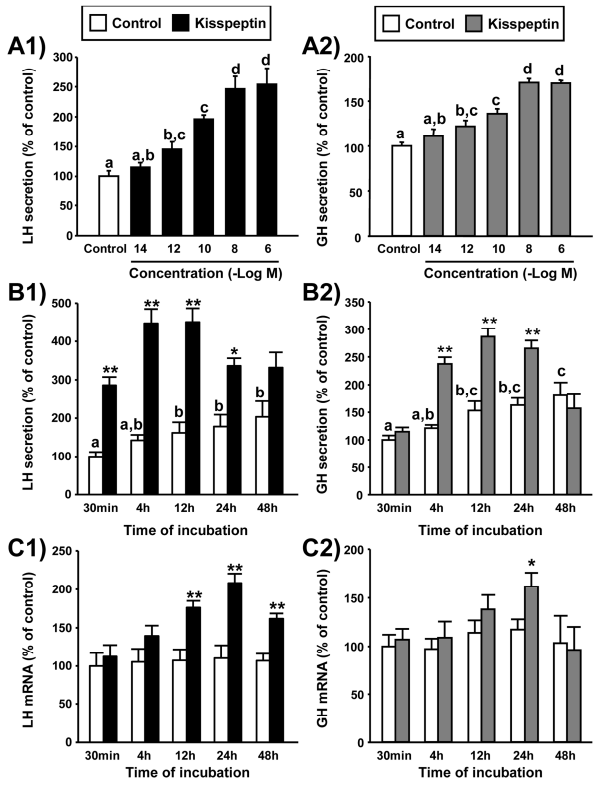

FIG. 1. Direct actions of kisspeptin-10 (Kp; 10nM) on baboon LH and GH synthesis and secretion: A) Effect of 4h treatment with Kp on LH (A1) and GH (A2) release. B) Time-dependent effect of Kp on LH (B1) and GH (B2) release. C) Time-dependent effect of Kp on *LH* (C1) and GH (C2) mRNA levels. Data are expressed as percent of control (set at 100%) at 4h (A) or 30 min (B and C). Values represent the mean ± SEM (n=4 individual experiments, 3-4 wells/expt). Values that do not share a common letter (a, b, c, d) are statistically different. Asterisks indicate values that significantly differ from their respective control values (same incubation time-period); *,p <0.05; **,p <0.01.

The direct actions of Kp on primate pituitary function were restricted to stimulation of LH and GH release, since this same experimental regime failed to significantly alter spontaneous FSH, PRL, ACTH and TSH release or their basal gene expression levels at any of the tested doses and time-points (Supplemental Fig. 1). The absolute impact of Kp on LH or GH secretion at a maximal dose (10nM; 4h-incubation) ranged between 2.2-4.9 fold for LH secretion and between 1.6-3.2 fold for GH release when compared with vehicle-treated controls, depending on the individual pituitary preparation (Supplemental Table 2). The magnitude of response did not correlate with age of the tissue donor, but may be associated with stage of the estrus cycle or metabolic environment, where this information was not available to us.

Interaction of Kp with major regulators of LH (GnRH) and GH (GHRH, Ghrelin and SST) release

Comparison of the effects of equimolar doses of Kp and GnRH revealed that both peptides induced similar increases of LH release, whereas Kp and GnRH co-administration elicited an additive increase in LH release, as compared to the effects of each peptide alone (Fig-2A). In contrast, Kp was less potent than GHRH and Ghrelin in inducing GH release *in vitro*, and only caused an additive stimulation on GH release in combination with GHRH, but not with ghrelin (Fig-2B). As expected, SST fully inhibited the stimulatory action of Kp on GH secretion (Fig-2B).

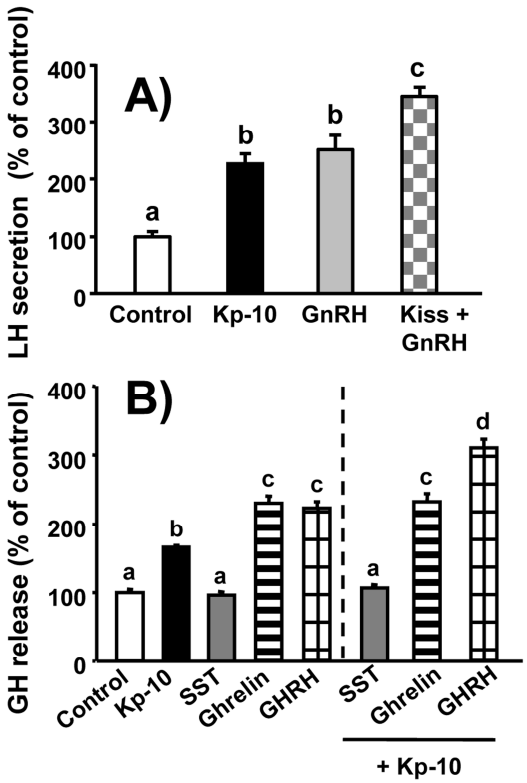

FIG. 2. Interaction of kisspeptin-10 (Kp; 10nM) with regulators of gonadotrope and somatotrope function in primary pituitary cell cultures from baboons. A) Effect of 4h treatment of Kp and/or GnRH (10 nM) on LH secretion. B) Effect of 4h treatment of Kp and/or GHRH, ghrelin (10 nM) or somatostatin (SST, 100nM) on GH secretion. Values are expressed as percentage of controls, set at 100% within each experiment, and represent the mean ± SEM of 4 independent experiments (3-4 wells/expt). Values that do not share a common letter (a, b, c, d) differ significantly (P < 0.05).

Intracellular signaling pathways involved in Kp-induced LH and GH release

Treatment of pituitary cell cultures with specific inhibitors of phospholipase C (PLC), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and intracellular Ca^{2+} mobilization, but not with blockers of adenylyl cyclase (AC), protein kinase A (PKA), extracellular Ca^{2+} influx (through L-type channels) or nitric oxide synthase (NOS), completely suppressed the stimulatory effects caused by Kp on baboon LH and GH release (Fig-3). Interestingly, blockade of mammalian target of Rapamycin (mTOR) or phosphoinositol 3-kinase (PI3K) activity completely abolished the stimulatory effect of Kp on LH secretion (Fig-3A), but not that of GH release (Fig-3B). Administration of these inhibitors alone did not modify basal LH or GH secretion (Fig-3).

In order to compare Kp- and GnRH-activated signaling pathways, a similar pharmacological approach was used following GnRH stimulation of pituitary cell cultures, which is also analogous to that employed in our previous reports documenting GHRH- and ghrelin-induced intracellular signals in pituitaries of baboons (23). However, owing to the limited availability of cell preparations, we were able to study only some selected baboon gonadotrophs (Supplemental Fig. 2).

Effect of estradiol (E2) on Kp-mediated LH and GH release

Sex steroids are key modulators of gonadotroph and somatotroph function. Thus, the influence of E2 on pituitary responsiveness to Kp in terms of LH and GH secretion was tested based on the previous experiments. Pre-incubation (36h) with 10 nM E2 increased spontaneous LH release by 63% (Fig-4A), and

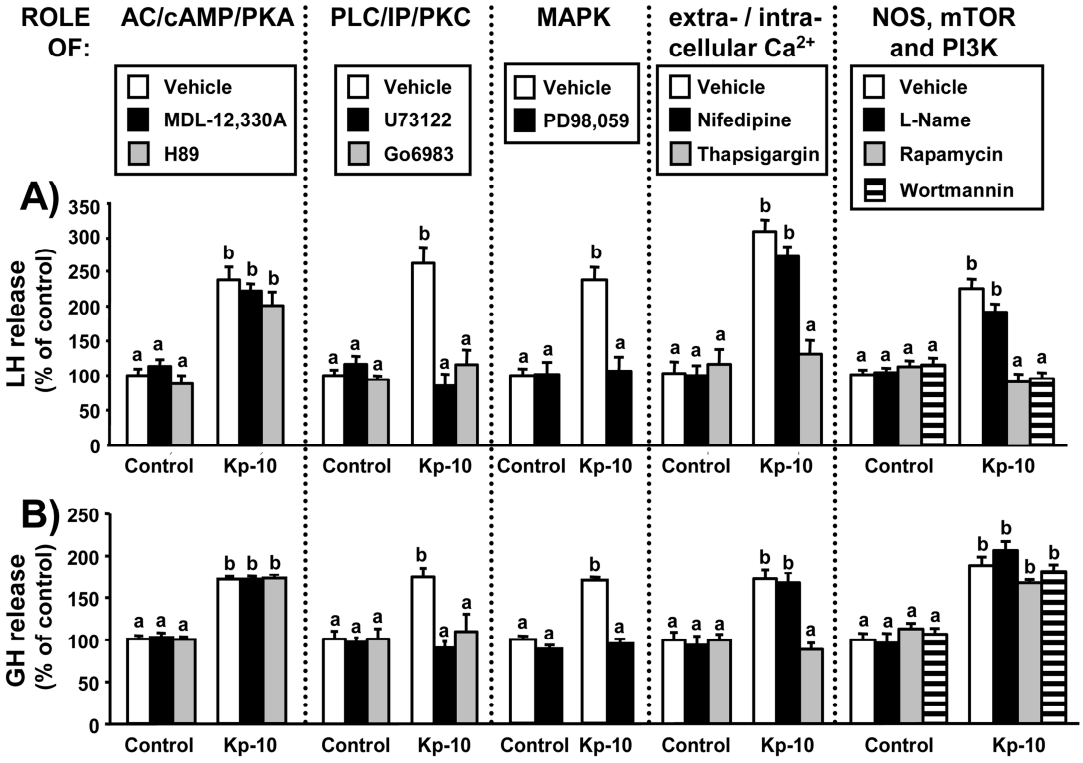

FIG. 3. Intracellular signaling pathways of kisspeptin-10 (kp)-stimulated baboon LH and GH release. Effect of the inhibition of AC (MDL-12,330A; 10 µM), PKA (H89; 15 µM), PLC (U73122; 50 µM), PKC (Go6983; 20 µM), MAPK (PD-98,059; 10 µM), extracellular Ca2+ L-type channels (nifedipine; 1 µM;), intracellular Ca2+ channels (thapsigargin; 10 µM), NOS (L-NAME; 10 µM), mTOR (Rapamycine; 10nM) and PI3K (Wortmannin; 1 µM) on kisspeptin-stimulated LH (A) and GH (B) release. On the day of the experiment, inhibitors were added to the incubation media 90min before Kp treatment (4 h; 10nM). Values are expressed as percentage of vehicle-treated controls without inhibitor (set at 100%) within each experiment, and represent the mean ± SEM of 3-5 independent experiments (3-4 wells/treatment/expt). Values that do not share a common letter (a or b) significantly differ ($P < 0.05$).

seemingly enhanced further the relative LH-releasing action of Kp administered alone or in combination with GnRH (Fig-4A). In contrast, pre-incubation with E2 decreased basal GH release from cultured somatotrophs by 40% (Fig-4B). Interestingly, while absolute stimulatory effects of GHRH and Ghrelin on GH release in the presence of E2 were concomitantly decreased, GH responses to Kp, either alone or in combination with GHRH (but not with ghrelin), were preserved in the presence of E2, despite the marked decrease in basal GH release, this resulting preserved in the presence of E2, despite the marked decrease in basal GH release, this resulting in an augmented capacity of somatotrophs to maintain its relative GH output response to Kp (Fig-4B).

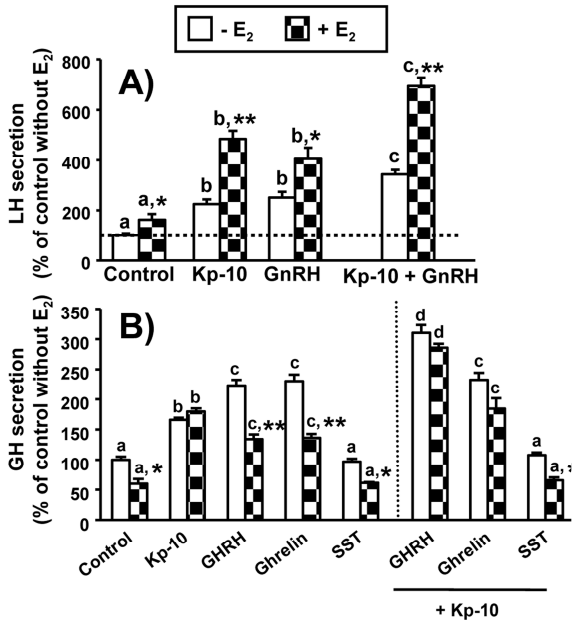

FIG. 4. Interaction of kisspeptin-10 (Kp; 10nM) with regulators of gonadotrope and somatotrope function in the absence or presence of estradiol (-E2 or +E2, respectively; 10nM) in primary pituitary cell cultures from baboons. A) Effect of 4h treatment of Kp and/or GnRH (10nM) on LH secretion. B) Effect of 4h treatment of Kp and/or GHRH, ghrelin (10nM) or somatostatin (SST; 100nM) on GH secretion. Data are expressed as percentage of controls without E2 (set at 100%), and represent the mean ± SEM of 3-4 independent experiments (3-4 wells/expt). Values that do not share a common letter (a, b, c, d) are statistically different. Asterisks indicate values that significantly differ from their respective control values (same treatment in the absence of estradiol); *,p <0.05; **,p <0.01.

In addition, the influence of E2 on the pattern of response to Kp of the remaining pituitary hormones was also evaluated (Supplemental Fig. 3). Similar to that observed for LH secretion, pre-incubation with E2 increased baseline FSH levels and uncovered an FSH-releasing effect of Kp. On the other hand, E2 did not alter basal PRL release; however, it sensitized lactotropes to positively respond to Kp. Finally, E2 failed to alter ACTH or TSH release, either in basal conditions or after Kp stimulation.

Discussion

The unexpected emergence in 2003 of Kps as novel, critical regulatory peptides for the reproductive axis imparted a renewed interest to the investigation of the neuroendocrine mechanisms underlying the control of gonadotropin secretion. Such an interest was boosted, among other ground-breaking findings, by the demonstration of the essential roles and extraordinarily potent gonadotropin-releasing effects of Kps in a wide variety of species, including rodents, sheep, monkeys and humans. Compelling evidence accumulated during the last years offers no hesitation as to the need of hypothalamic Kps and *Kiss1r* to attain -and maintain- proper gonadotropin secretion and reproductive capacity. Yet, the paramount importance of their hypothalamic actions might have obscured more subtle, albeit discernable, actions of Kps at other levels of the gonadotropic axis. Thus, in spite of early reports showing high levels of expression of *Kiss1r* in the pituitary, the number of functional studies directed at understanding the role and mechanism of action of Kps on pituitary hormone secretion are limited. Therefore, our present study provides comprehensive experimental evidence to support a plausible role of Kps in the direct pituitary control of gonadotroph (and somatotroph) function in the baboon, a primate model of likely interest for human physiology and translational medicine (23, 25-28).

It is well known that a number of central and peripheral factors can act directly on the pituitary to regulate the release of hypophyseal hormones either independently of, or cooperatively with, their putative hypothalamic drivers, e.g. ghrelin also stimulates GH from primate somatotrophs in a GHRH-independent manner (23). In line with this notion, mRNA expression and/or presence of proteins for both *Kiss1r* and *Kiss1* have been

described in the pituitary of humans, rodents, bovine and ovine (10, 15-17, 29); and Kp has been shown to induce LH release in pituitary cultures of several species (9, 10, 21). In spite of this latter evidence, the actual biological significance of this system in the pituitary still remains controversial, as contradictory results have also been reported (7, 18, 19). In the present work, we demonstrate that Kp-10 is able to directly stimulate *specific* populations of baboon pituitary cells *in vitro*. First, and most notably, Kp-10 significantly stimulated LH synthesis and release in a dose-dependent manner in cultured gonadotrophs. Moreover, this stimulation was additive to the GnRH action, suggesting the possibility that the pathways mediating LH release in response to GnRH and Kp at the gonadotroph level may be partially independent. Consistent with this, *Kiss1r* expression has been described in gonadotophs (12, 17) and immunocytochemical studies in rat pituitary have shown co-localization of LHß and *kiss1r* which would enable and support a direct action of Kp on gonadotrophs(17), as it is also substantiated by the ability of Kp-10 to evoke direct calcium responses from individual rat gonadotrophs in culture (10). Furthermore, our present data also demonstrate that the stimulation of LH elicited by Kp-10 upon *Kiss1r* activation at the pituitary is mediated by mTOR, PI3K, MAPK, PLC and intracellular Ca^{2+} mobilization, a complex set of second messenger pathways which remarkably parallels that found previously to mediate the actions of *kiss1r* on GnRH neurons (11, 30, 31). Interestingly, it should be noted that under the experimental conditions employed in this study, Kp-10 failed to alter FSH release. Although LH and FSH are known to be co-stored in a subpopulation of bihormonal gonadotrophs, and are often co-regulated and released in response to diverse stimuli (32), it has also been shown in a number of studies that FSH release can follow a markedly different pattern than that of LH. For example, the frequency of GnRH can differentially affect LH and FSH release (33). Therefore, it is possible that the pattern of delivery of Kp to gonadotropes in vitro, may also differentially regulate LH and FSH release. It should also be noted that preincubation of E2 unmasked a stimulatory effect of Kp on FSH release, therefore it is possible that the steroidal milieu may differently influence LH and FSH response to Kps in vitro under the culture conditions employed herein.

In addition to its effects on LH secretion, Kp-10 also induced GH synthesis and release, at doses equivalent to those effective for LH secretion. This stimulation, however, was of lower magnitude than that evoked by the primary GH secretagogues, GHRH and ghrelin. Noteworthy, Kp-dependent GH release is additive to GHRH stimulation but not with ghrelin action. Specifically, co-administration of Kp-10 and GHRH leads to a additive effect, analogous to that previously observed after co-administration of ghrelin and GHRH (23). Interestingly, the set of second messenger pathways required by Kp-10 to stimulate GH release seems to be more limited than that required for LH release, involving PLC and MAPK activation and intracellular Ca^{2+} mobilization. This divergence in *kiss1r*-mediated signaling suggests that Kp-10 directly activates a distinct population of pituitary cells to induce GH release, presumably somatotrophs, which have been proposed to express *kiss1r* as well (12, 17, 23).

An important question arising from our data is to what extent the present observations represent a physiologically relevant phenomenon in a species, such as the baboon, of particular interest as a model to human biology (23, 25-28). Our present observations extend and refine previous findings describing the ability of Kp to directly elicit LH secretion at the pituitary in other species; this might be a contributing, fine-tuning mechanism for the well-characterized ability of Kps to potently stimulate gonadotropin release in primates. On the other hand, our results are suggestive of putative regulatory actions of Kps on other pituitary axes, such as the somatotrophic system. Admittedly, while the capacity of Kp to potently activate gonadotropin secretion (mainly through central mechanisms) is indisputable, its action on GH release remains controversial. Thus, while a number of *in vitro* studies have documented a significant stimulation of GH (10, 21), several *in vivo* studies, performed recently in cattle, pigs and other primates (*Macaca mulatta*), did not observe this effect (7, 34, 35). In contrast, work by Kadokawa et al. documented a stimulatory action of Kp (i.v.) on GH release in Holstein heifers (20), supporting the somatotrope action of Kp described by the *in vitro* assays. Of

note, mice lacking a functional *Kiss1r* gene (*Kiss1r* Knockout) show a significant reduction in body weight in adulthood (36); yet, whether this effect is due to impairments in GH release during development and/or to the absence of the anabolic effect of sex steroids, or other factors remains to be elucidated. A possible explanation for the differential results between *in vitro* and *in vivo* studies might be related to the lower sensitivity of pituitary cells to Kp (pM range), compared to GnRH neurons where Kp has been proven to be effective in the fM range (11). Therefore, it is not unreasonable to think that peripherally-injected Kp-10, which is considerably short-lived, might not achieve concentrations high enough to stimulate somatotrope function. Of note, the main source of Kps acting on the pituitary could be local, since *Kiss1* and Kp have been described in gonadotrophs, somatotrophs and lactotrophs (12). However, it should be noted that Kps are detectible in hypophysial portal blood in ewes (12), and kisspeptin-54 release have been reported to occur *in vivo* in the stalk-median eminence of female rhesus monkeys at the time of the pubertal increase in GnRH release (37). Taken together this data suggest that centrally derived Kps may reach their putative pituitary cell targets at sufficient quantities to exert their direct actions; an issue that certainly deserves investigation.

In addition, our results demonstrate that sex steroids modulated the impact of *kiss1* on the pituitary. Specifically, E2 enhanced the stimulatory action of Kp-10 upon gonadotropin and prolactin release (the latter exclusively dependent on the presence of E2). Regulation of *Kiss1* expression by E2 in the pituitary has been previously documented in rodent and bovine (9, 17), and coincides with the regulation of *Kiss1* expression at the AVPV of female rodents (4). Importantly, gonadotroph-specific estrogen receptor-α knockout mice are infertile due to impairments in LH and FSH release (38), pointing to a crucial role for estradiol signaling at the gonadotrophs in the control of reproductive function, which might putatively involve its capacity to enhance Kp-induced gonadotropin secretion directly at the pituitary level. In line with this, E2 has been reported to enable kisspeptin-mediated increases in GABA and glutamate transmission to stimulate GnRH neurons (39), and to enhance GnRH/gonadotropin responsiveness to Kp-10 in rodents (40, 41). Moreover, results generated by other laboratories support a facilitative role of E2 acting on the pituitary to increase LH release in response to GnRH (42, 43), which is comparably observed in the present study. The molecular mechanism underlying this enhanced GnRH action seems to involve an increased E2-induced cAMP level (43). In the current study, data is not available as to whether cAMP levels and/or, for instance, increased *Kiss1r* expression, could mediate the E2-induced Kp10 enhanced stimulatory action. Although previous studies did not show any variation in *Kiss1r* expression in relation to sex steroid milieu at the hypothalamic level in rats (44), recent work from our group suggest that E2 may enhance the ability of kp10 to augment
Kiss1r expression from cultured pituitary cells (our unpublished data). Nevertheless, further studies will be required to clarify this issue. In any case, our current data further extend those original observations, suggesting that such facilitative action of E2 on the Kp-mediated control of gonadotropin secretion might include a pituitary site of action as well. In fact, E2 also influences somatotroph responsiveness to Kp, although in a manner distinct from that observed for gonadotropes. Specifically, the presence of E2 maintained Kp-induced GH output, but markedly reduced basal GH release and blunted the GH response to the primary secretagogues, GHRH and ghrelin. The mechanisms behind these actions, as well as its physiological relevance, are yet to be elucidated, but the action on different pituitary cell populations and the apparent recruitment of distinct subsets of intracellular signaling systems might help to explain such divergence in E2 effects.

In sum, we report for the first time a detailed description of the stimulatory effect of Kps at the pituitary level in the female baboon. Our results add further, convincing evidence to reinforce previous observations obtained in non-primate species, and strongly suggest that, besides its major central actions, direct effects of Kps at the pituitary may represent an additional level of regulation in the control of hypophyseal hormone (prominently, LH) release. In addition, our study offers new insights on the molecular mechanisms whereby Kps elicit LH (and GH) secretion directly at the pituitary level, in a species of special

interest for human physiology and translational medicine.

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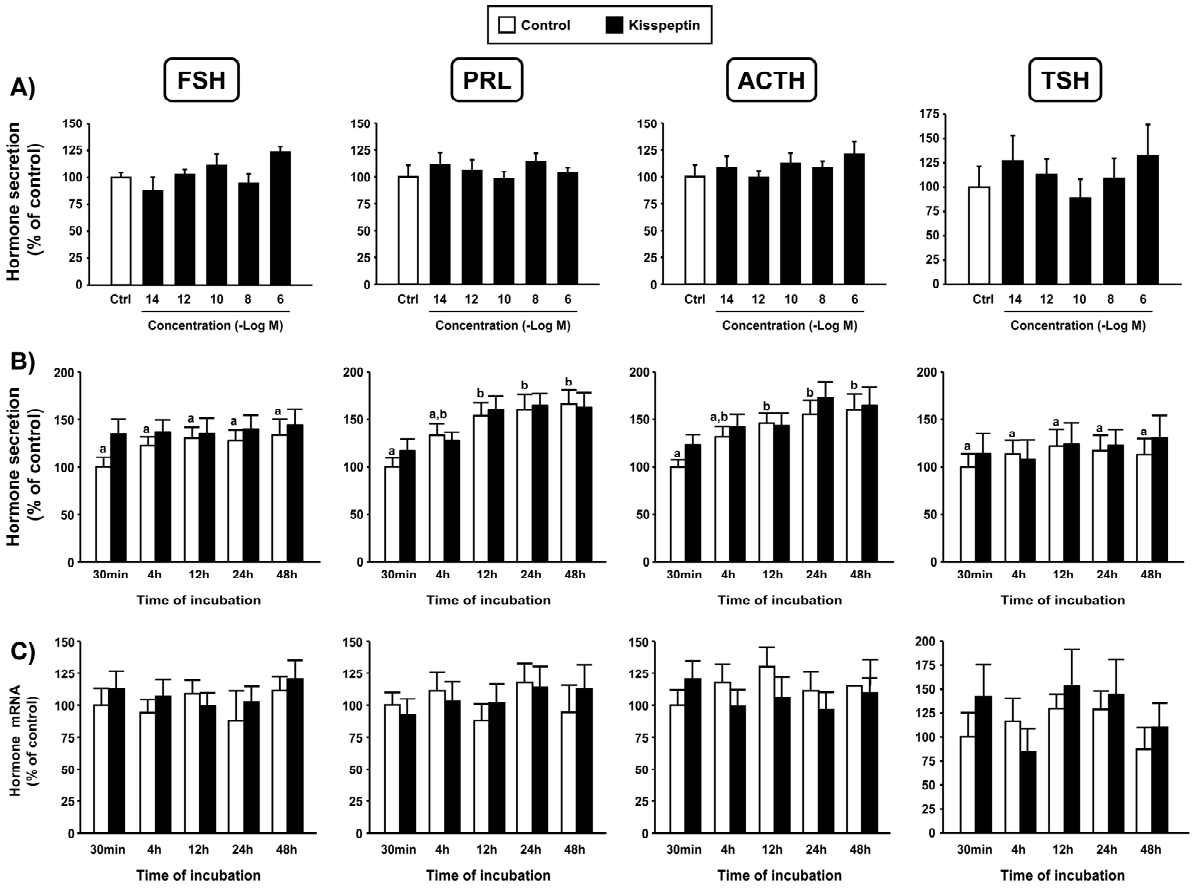
Supplemental Table 1: Baboon-specific primers for amplification of transcripts of LH, GH, FSH, ACTH, PRL, TSH, and cyclophilin A used for qrtRT-PCR.

Gene	Genbank Accession #	Primer Sequence	Nucleotide Position	Product Size
LH	HQ012663	**Sense**: GCCTCCTCTTCCTCTAAAGACC **Antisense**: GCGGATTGAGAAGCCTTTATT	**Sn** 59 **As** 162	104
GH	DQ340390	**Sense**: GACCTAGAGGAAGGCATCCAAA **Antisense**: AGCAGCCCGTAGTTCTTGAGTAG	**Sn** 21 **As** 163	143
FSH	HQ012664	**Sense**: TTGGTGTGCTGGCTACTGCT **Antisense**: GGGCACTCTCACTGTTTCGT	**Sn** 96 **As** 210	115
POMC	DQ315472	**Sense**: CCCTACAGGATGGAGCACTT **Antisense**: CGTTCTTGATGATGGCGTTT	**Sn** 7 **As** 133	127
PRL	EF419886	**Sense**: CCTTCGAGACCTGTTTGACC **Antisense**: ATCTGTTGGGCTTGCTCCTT	**Sn** 12 **As** 194	183
TSH	HQ012665	**Sense**: ATTGCCTAACCATCAACACCAC **Antisense**: AAACATCCTGGGACAGAGCATA	**Sn** 59 **As** 160	102
Kiss1r	HQ012666	**Sense**: CCAACTTCTACATCGCCAACC **Antisense**: ACATGAAGTCGCCCAGCA	**Sn** 29 **As** 142	114
Cyclophilin A	DQ315473	**Sense**: CAAGACGGAGTGGTTGGATG **Antisense**: TGGTGGTCTTCTTGCTGGTC	**Sn** 351 **As** 472	122

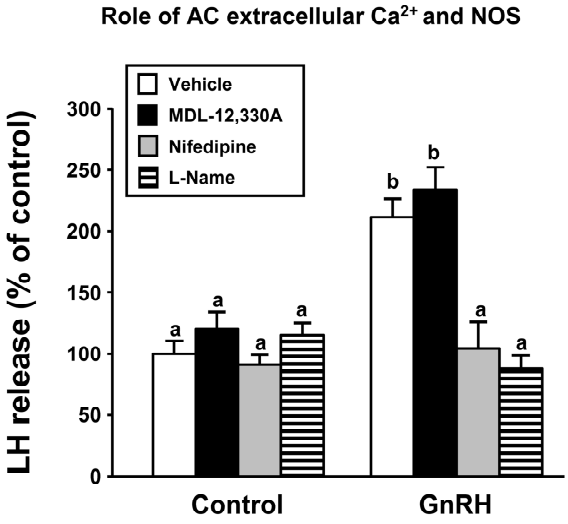
Supplemental Table 2: Kisspeptin-10 (Kp)-stimulated luteinizing-hormone (LH) and growth-hormone (GH) release vs. vehicle treated controls. Values represent the percentage of Kp (10nM; 4h-incubation)-stimulated LH or GH release vs. vehicle treated controls (set at 100%) at 4h. Values represent the mean ± SEM of individual baboon primary pitutary cell cultures (3-4 wells/expt).

			Kp-stimulated hormone release vs. vehicle treated control (set at 100%)	
	Age		**LH**	**GH**
Baboon #	**Years**	**Months**	**Mean SEM**	**Mean SEM**
1	7	2	379 ± 41	288 ± 16
2	7	7	374 ± 28	177 ± 5
3	7	9	292 ± 36	322 ± 21
4	8	3	289 ± 21	159 ± 7
5	8	8	289 ± 44	189 ± 11
6	8	12	407 ± 62	312 ± 19
7	9	6	413 ± 38	169 ± 6
8	9	6	493 ± 52	165 ± 8
9	9	10	447 ± 36	172 ± 6
10	11	3	221 ± 13	196 ± 10
11	11	9	316 ± 22	170 ± 4

Supplemental Figure 1: A) Direct effect of 4h treatment with kisspeptin-10 on baboon FSH, PRL, ACTH and TSH secretion. B) Time-dependent effect of Kisspeptin (10nM) on FSH, PRL, ACTH and TSH release. C) Time-dependent effect of Kisspeptin-10 (10nM) on FSH, PRL, ACTH and TSH mRNA levels in primary pituitary cell cultures from baboons. Data are expressed as percent of control (set at 100%) at 4h (A) or 30 min (B and C). Values represent the mean ± SEM (n = 4 individual experiments, 3-4 wells/expt). Values that do not share a common letter (a or b) are statistically different ($p < 0.05$).



Supplemental Figure 2: Effect of inhibition of AC (MDL-12,330A; 10 µM; n=2), extracellular Ca2+ channels (nifedipine; 1 µM; n=2) and NOS (L-NAME;10 µM; n=1) on GnRH-stimulated LH release. On the day of the experiment, inhibitors were added to the incubation media 90 min before kisspeptin treatment (4 h; 10nM). Values are expressed as percentage of vehicle-treated controls without inhibitor (set at 100%) within each experiment, and represent the mean ± SEM of 1-2 independent experiments (three to four wells per treatment per experiment). Values that do not share a common letter (a or b) significantly differ ($P < 0.05$).



Supplemental Figure 3: Effect of estradiol (+E2) on basal or kisspeptin-induced FSH, PRL, ACTH and TSH release in baboon primary pituitary cell cultures. Data are expressed as percentage of controls without E2 (-E2; set at 100%), and represent the mean ± SEM of 3-4 independent experiments (3-4 wells/expt). Values that do not share a common letter (a, b, c) are statistically different ($p < 0.05$).